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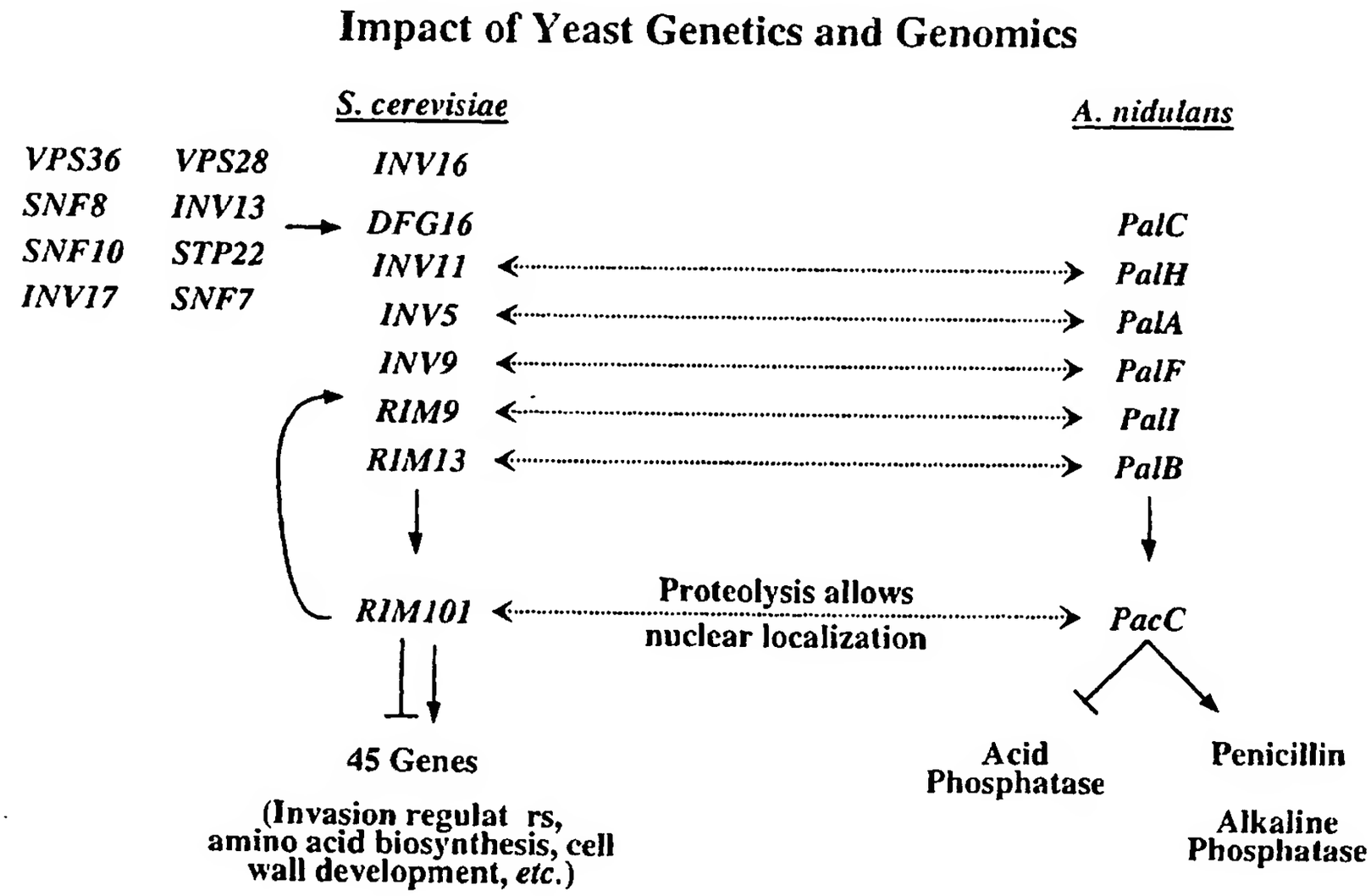
(54) Title: METHODS FOR IMPROVING SECONDARY METABOLITE PRODUCTION IN FUNGI



(57) Abstract: The invention relates to the production of secondary metabolites by fungi. More particularly, the invention relates to improvement of production of commercially important secondary metabolites by fungi. The invention provides methods for improving secondary metabolite production in a fungus, comprising modulating the expression of a gene involved in regulation of secondary metabolite production.

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METHODS FOR IMPROVING SECONDARY METABOLITE PRODUCTION IN FUNGI

BACKGROUND OF THE INVENTION

Field of the invention

The invention relates to the production of secondary metabolites by fungi. More particularly, the invention relates to improvement of production of commercially important secondary metabolites by fungi.

Summary of the related art

Secondary metabolite production by various fungi has been an extremely important source of a variety of therapeutically significant pharmaceuticals. B-lactam antibacterials such as penicillin and cephalosporin are produced by *Penicillium chrysogenum* and *Acremonium chrysogenum*, respectively, and these compounds are by far the most frequently used antibacterials (reviewed in Luengo and Penalva, Prog. Ind. Microbiol. 29: 603-38 (1994); Jensen and Demain, Biotechnology 28: 239-68 (1995); Brakhage, Microbiol. Mol. Biol. Rev. 62: 547-85 (1998)). Cyclosporin A, a member of a class of cyclic undecapeptides, is produced by *Tolypocladium inflatum*. Cyclosporin A dramatically reduces morbidity and increases survival rates in transplant patients (Borel, Prog. Allergy 38: 9-18 (1986)). In addition, several fungal secondary metabolites are cholesterol lowering drugs, including lovastatin that is made by *Aspergillus terreus* and several other fungi (Alberts *et al.*, Proc. Natl. Acad. Sci. USA 77: 3957-3961 (1980)). These and many other fungal secondary metabolites have contributed greatly to health care throughout the world (see Demain, Ciba Found Symp 171: 3-16 (1992); Bentley, Crit. Rev. Biotechnol. 19: 1-40 (1999)).

Unfortunately, many challenges are encountered between the detection of a secondary metabolite activity to production of significant quantities of pure drug. Thus, efforts have been made to improve the production of secondary metabolites by fungi. Some of these efforts have attempted to improve production by modification of the growth medium or the bioreactor used to carry out the fermentation. Buckland *et al.*, in Topics in Industrial Microbiology: Novel Microbial products for Medicine and Agriculture, pp. 161-169, Elsevier, Amsterdam (1989)

discloses improved lovastatin production by modification of carbon source and also teaches the superiority of a hydrofoil axial flow impeller in the bioreactor. Other efforts have involved strain improvements, either through re-isolation or random mutagenesis. Agathos *et al.*, J. Ind. Microbiol. 1: 39-48 (1986), teaches that strain improvement and process development together resulted in a ten-fold increase in cyclosporin A production. While important, studies of these types have still left much room for improvement in the production of secondary metabolites.

More recently, strains have been improved by manipulation of the genes encoding the biosynthetic enzymes that catalyze the reactions required for production of secondary metabolites. Penalva *et al.*, Trends Biotechnol. 16: 483-489 (1998) discloses that production strains of *P. chrysogenum* have increased copy number of the penicillin synthesis structural genes. Other studies have modulated expression of other biosynthetic enzyme-encoding genes, thereby affecting overall metabolism in the fungus. Mingo *et al.*, J. Biol. Chem. 21: 14545-14550 (1999), demonstrate that disruption of phacA, an enzyme in *A. nidulans* that catalyzes phenylacetate 2-hydroxylation, leads to increased penicillin production, probably by elimination of competition for the substrate phenylacetate. Similarly, disruption of the gene encoding aminoadipate reductase in *P. chrysogenum* increased penicillin production, presumably by eliminating competition for the substrate alpha-aminoadipate (Casquiero *et al.*, J. Bacteriol. 181: 1181-1188 (1999)).

Thus, genetic manipulation holds promise for improving production of secondary metabolites. Genetic manipulation to increase the activity of biosynthetic enzymes for secondary metabolite production or to decrease the activity of competing biosynthetic pathways has proven effective for improving production. Maximum benefit might be achieved by combining several strategies of manipulation. For example, modulating the expression of genes that regulate the biosynthetic enzyme-encoding genes might improve production. In addition, genetic manipulation could be used to impact upon the challenges that are encountered in the fermentor run or downstream processing (*e.g.* energy cost, specific production of desired metabolite, maximal recovery of metabolite, cost of processing waste from fermentations). There is, therefore, a need for methods for improving secondary metabolite production in a fungus, comprising modulating the expression of a gene involved in regulation of secondary metabolite production. Ideally, such methods should be able to provide increased yield, increased

productivity, increased efflux/excretion, decreased production of side products or non-desired secondary metabolites, altered strain characteristics and/or conditional lysis, or increased resistance to the deleterious effects of exposure to a secondary metabolite.

BRIEF SUMMARY OF THE INVENTION

The invention provides methods for improving secondary metabolite production in a fungus, comprising modulating the expression of a gene involved in regulation of secondary metabolite production. The methods according to the invention provide increased yield, increased productivity, increased efflux/excretion, decreased production of side products or non-desired secondary metabolites, altered strain characteristics and/or conditional lysis, or increased resistance to the deleterious effects of exposure to a secondary metabolite.

The several aspects of the methods according to the invention are preferably achieved by overexpression of regulatory genes, expression of dominant mutations, use of peptide activators or inhibitors, use of small molecule activators or inhibitors, and conditional expression of regulatory genes. These factors preferably modulate transcription factors, transmembrane transporters, proteins that mediate secretion, kinases, G-proteins, cell surface receptors, GTPase activating proteins, guanine nucleotide exchange factors, phosphatases, proteases, phosphodiesterases, bacterial protein toxins, importins, RNA-binding proteins, SCF complex components, adherins, or biosynthetic pathways.

The invention further provides for achieving the aspects described in the invention by combinatorial manipulation. Combinatorial manipulation is the simultaneous use of multiple methods and/or multiple factors to achieve the aspects of the invention. Methods for achieving the aspects of the invention are preferably by the overexpression of regulatory genes, expression of dominant mutations, use of peptide activators or inhibitors, use of small molecule activators or inhibitors, and conditional expression of regulatory genes. The preferred factors are as described above.

The invention further provides genetically modified fungi, wherein the genetically modified fungi have an ability to produce secondary metabolites and the ability of the genetically modified fungus to produce secondary metabolites has been improved by any of the methods according to the invention.

The invention also provides a method for making a secondary metabolite, the method comprising culturing a genetically modified fungus according to the invention under conditions suitable for the production of secondary metabolites.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the ability of PUMP1 (AAD34558) from *Aspergillus terreus* to confer lovastatin resistance to a yeast strain.

Figure 2 shows the impact of yeast genetics and genomics on fungal genetics. Arrows indicate which genes or gene products act on other genes or gene products.

Figure 3 shows representative box plot presentations of lovastatin production data from shake flask experiments. Data from strains that express a particular regulator (*e.g.*, pacCVP16) are displayed with appropriate negative (EMPTY or GUS) and positive (lovE) controls from the same shake flask experiment.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention relates to the production of secondary metabolites by fungi. More particularly, the invention relates to improvement of production of commercially important secondary metabolites by fungi. The references cited herein evidence the level of knowledge in the field and are therefore incorporated by reference in their entirety. In the event of a conflict between a cited reference and the present specification, the latter shall prevail.

The invention provides methods for improving secondary metabolite production in a fungus, comprising modulating the expression of a gene involved in regulation of secondary metabolite production. In certain embodiments, the methods comprise modulating the expression of more than one gene involved in regulation of secondary metabolite production.

The experiments disclosed in this specification demonstrate how genetic manipulation can be employed to improve the process of secondary metabolite production in fungi. In these experiments, strains have been manipulated to express fungal regulators, and in many instances these modifications resulted in improvements such as increased yield of metabolite, increased productivity of metabolite, or beneficial morphological and behavioral characteristics. These manipulations have improved production of secondary metabolites, including both the β-lactam antibiotic penicillin as well as the polyketide anti-hypercholesterolemic drug lovastatin. The discovery that this engineering (of regulatory pathways instead of biosynthetic genes) can improve the production of multiple classes of drugs together with the demonstration that this process can be implemented in various fungi suggests that the process of genetic manipulation of regulators of secondary metabolism will be a general tool for improving secondary metabolite production in fungi.

In a first aspect, the invention provides methods for improving production of a secondary metabolite by a fungus by increasing the yield of the secondary metabolite produced by the fungus. The methods according to this aspect of the invention comprise modulating the expression of a gene involved in regulation of secondary metabolite production in a manner that improves the yield of the secondary metabolite.

Preferably, for this aspect of the invention, when the secondary metabolite is isopenicillin N, then the modulation is not mediated by the transcription factor CPCR1; when the secondary metabolite is sterigmatocystin, then the modulation is not through AflR, FadA, or FluG; when the secondary metabolite is aflatoxin, then the modulation is not through AflR; when the secondary metabolite is penicillin and the fungus is *Aspergillus nidulans*, then the modulation is not through mutations that result in expression of truncated forms of PacC or constitutively active forms of FadA; when the gene involved in regulation of secondary metabolite production is from *Saccharomyces cerevisiae*, then the modulation is not through decreased activity or expression of BEM2, HOG1, IRA1, RIM15, SFL1, SRB11, SSD1, SWI4, TPK3 or though increased activity or expression of AFL1, CDC25, DHH1, HAP4, INV11, INV13, INV5, INV7, INV9, MCM1, MEP2, MGA1, MSN1, MSN5, MSS11, PET9, PHO23, PTC1, RIM101, RIM13, RIM9, SNF8, STP22, TPK2, VPS28, VPS36, or YPR1.

As used for all aspects of the invention, the term "improving production of a secondary metabolite" means to positively impact upon one or more of the variables that affect the process of production of secondary metabolites in a fungal fermentation. These variables include, without limitation, amount of secondary metabolite being produced, the volume required for production of sufficient quantities, the cost of raw materials and energy, the time of fermentor run, the amount of waste that must be processed after a fermentor run, the specific production of the desired metabolite, the percent of produced secondary metabolite that can be recovered from the fermentation broth, and the resistance of an organism producing a secondary metabolite to possible deleterious effects of contact with the secondary metabolite. Also for all aspects, the term "secondary metabolite" means a compound, derived from primary metabolites, that is produced by an organism, is not a primary metabolite, is not ethanol or a fusel alcohol, and is not required for growth under standard conditions. Secondary metabolites are derived from intermediates of many pathways of primary metabolism. These pathways include, without limitation, pathways for biosynthesis of amino acids, the shikimic acid pathway for biosynthesis of aromatic amino acids, the polyketide biosynthetic pathway from acetyl coenzyme A (CoA), the mevalonic acid pathway from acetyl CoA, and pathways for biosynthesis of polysaccharides and peptidopolysaccharides. Secondary metabolism involves all primary pathways of carbon metabolism (Fungal Physiology, Chapter 9 pp. 246-274 ed. DH Griffin (1994)). "Secondary

metabolites" also include intermediate compounds in the biosynthetic pathway for a secondary metabolite that are dedicated to the pathway for synthesis of the secondary metabolite. "Dedicated to the pathway for synthesis of the secondary metabolite" means that once the intermediate is synthesized by the cell, the cell will not convert the intermediate to a primary metabolite. "Intermediate compounds" also include secondary metabolite intermediate compounds which can be converted to useful compounds by subsequent chemical conversion or subsequent biotransformation. Nevertheless, providing improved availability of such intermediate compounds would still lead to improved production of the ultimate useful compound, which itself may be referred to herein as a secondary metabolite. The yeast *Saccharomyces cerevisiae* is not known to produce secondary metabolites. The term "primary metabolite" means a natural product that has an obvious role in the functioning of almost all organisms. Primary metabolites include, without limitation, compounds involved in the biosynthesis of lipids, carbohydrates, proteins, and nucleic acids. The term "increasing the yield of the secondary metabolite" means increasing the quantity of the secondary metabolite present in the fermentation broth per unit volume of fermentation broth.

A "gene involved in regulation of secondary metabolite production" is a gene, other than a gene encoding a biosynthetic enzyme, which modulates secondary metabolite production involving yield, productivity, efflux/excretion, production of side products or non-desired secondary metabolites, strain characteristics and/or conditional lysis, or resistance to the deleterious effects of exposure to a secondary metabolite. A "biosynthetic enzyme" is a molecule that catalyzes the conversion of a substrate to a product, including an intermediate product, in a biosynthetic pathway for a secondary metabolite.

As used for all aspects of the invention, the term "modulating the expression of a gene" means affecting the function of a gene's product, preferably by increasing or decreasing protein activity through mutation, creating a new protein activity through mutation; increasing or decreasing transcription, increasing or decreasing translation, increasing or decreasing post-translational modification, altering intracellular localization, increasing or decreasing translocation, increasing or decreasing protein activity by interaction of the protein with another molecule, or creating a new protein activity by interaction of the protein with another molecule. In some cases, such modulation is achieved simply by allowing or causing the expression of an

exogenously supplied nucleic acid or gene. In some cases other exogenously supplied molecules may mediate the modulation. The modulation is not achieved, however, by simply randomly mutagenizing the fungus, either spontaneously or by chemical means.

As used for all aspects of the invention, "mutation" means an alteration in DNA sequence, either by site-directed or random mutagenesis or by recombination. Mutation encompasses point mutations as well as insertions, deletions, or rearrangements.

As used for all aspects of the invention, "mutant" means an organism containing one or more mutations.

In certain embodiments of the methods according to this aspect of the invention, the modulation is overexpression of the gene. "Overexpression of the gene" means transcription and/or translation and/or gene product maturation at a rate that exceeds by at least two-fold, preferably at least five fold, and more preferably at least ten-fold, the level of such expression that would be present under similar growth conditions in the absence of the modulation of expression of the gene. "Similar growth conditions" means similar sources of nutrients such as carbon, nitrogen, and phosphate, as well as similar pH, partial oxygen pressure, temperature, concentration of drugs or other small molecules, and a similar substrate for growth, whether solid, semi-solid, or liquid. Preferred genes according to this aspect of the invention include, without limitation, AAD34561, abaA, ACE2, ADR1, AFL1, aflR, AFT1, amyR, areA, ASH1, BAP2, BCY1, CAT8, CDC24, CDC25, CDC28, CDC42, CDC55, CLB2, creA, CTS1, CUP9, CYR1, DFG16, DHH1, DPH3, ELM1, facB, FLO1, FLO11, FLO8, FUS3, GCN2, GCN4, GCR1, GCR2, GLN3, GPA1, GPA2, GPR1, GRR1, GTS1, HAP1, HAP4, HIP1, HMS1, HMS2, HOG1, HSL1, HXK2, IME1, IME4, INO2, INV11, INV13, INV16, INV5, INV7, INV9, KSS1, LEU3, lovE, LYS14, MAC1, MCM1, MEP1, MEP2, MET28, MET31, MET4, metR, MGA1, MIG1, MIG2, MSN1, MSN2, MSN4, MSN5, MSS11, MTH1, NPR1, nreB, NRG1, OAF1, pacC, PBS2, PDE2, PET9, PHD1, PHO2, PHO4, PHO85, pkaR, PPR1, PTC1, PUT3, RAS1, RAS2, RGS2, RIM101, RIM13, RIM15, RIM9, ROX1, RRE1, SCH9, sconB, SFL1, SHO1, SHR3, SIN3, SIP4, SKN7, SNF1, SNF2, SNF7, SNF8, SOK2, SRB10, SRB11, SRB8, SRB9, sreA, sreP, SRV2, SSD1, SSN6, SST2, STE11, STE12, STE20, STE50, STE7, STP22, SWI4, SWI6, tamA, TEC1, TPK1, TPK2, TPK3, TUP1, UaY, UGA3, URE2, VPS28, VPS36, WHI3,

YMR077c, YNL255c, YPR1, ZAP1, genes encoding bacterial protein toxins, and any fungal homologs of the aforementioned genes.

In certain embodiments of the methods according to this aspect of the invention, the modulation is expression of a dominant mutation of the gene. A "dominant mutation" is an allele of a gene that encodes a protein capable of changing the phenotype of an organism more than a non-mutated form of the gene. Preferred dominant mutations include dominant negative mutations, dominant positive mutations, and dominant neomorphic mutations. A "dominant negative mutation" is a dominant mutation that achieves its phenotypic effect by interfering with some function of the gene or gene product from which it was derived, or from a homolog thereof. A "dominant positive mutation" is a dominant mutation that achieves its phenotypic effect by activating some function of the gene or gene product from which it was derived, or from a homolog thereof. A "dominant neomorphic mutation" is a dominant mutation that achieves the phenotypic effect of providing a novel function to the gene or gene product from which it was derived, or from a homolog thereof. Preferred dominant mutations according to this aspect of the invention include:

1. Mutations that result in increased or decreased stability of the transcript of a gene.
2. Mutations that result in increased or decreased stability of the product of translation: For example, specific sequences near the amino terminus of a protein have been shown to cause increased or decreased protein stability. Similarly, sequences elsewhere in the protein, such as those required for ubiquitin-dependent degradation, can be mutated to increase the stability of a protein.
3. Amino acid substitutions that mimic post-translational modifications: For example, phosphorylation has been demonstrated to positively or negatively regulate the activity of a variety of proteins, including transcription factors and kinases. Phosphorylation most commonly occurs on serine, threonine, and tyrosine residues; in some instances residues such as aspartate and histidine can be phosphorylated. Mutations that mimic constitutive dephosphorylation can be produced by mutating the coding sequence of the phosphorylated residue to the coding sequence of an amino acid that cannot be phosphorylated and does not have a negatively charged side chain (e.g. alanine). Alternatively, substitutions that result in changing serine, threonine, or

tyrosine residues to charged amino acids such as glutamate or aspartate can result in an allele that mimics constitutive phosphorylation.

Proteolytic cleavage is another post-translation mechanism for regulating the activity of a protein. Mutations that result in truncation of a protein might mimic activation by proteolysis. Mutations that change amino acids required for proteolysis could activate proteins that are negatively regulated by proteolysis.

4. Amino acid substitutions that promote or inhibit the binding of small molecules such as ATP, cAMP, GTP or GDP: For example, ATP is a co-factor for many enzymatic reactions, and the nucleotide-binding domains of these proteins are highly conserved. Lysine to arginine substitutions in the nucleotide binding domain frequently result in inhibition of enzymatic activity. Enzymatically inactive proteins could be dominant inactive molecules, acting by sequestering substrates from functional enzymes.

cAMP is required for the activation of protein kinase A. Protein kinase A consists of regulatory subunits and catalytic subunits. The binding of cAMP to the negative regulatory subunit relieves its inhibition of the catalytic subunit. Therefore, mutations that prevent cAMP binding could result in constitutive inactivation of protein kinase A.

G-proteins are a class of proteins that bind the nucleotides GTP and GDP. The GTP-bound form of these proteins is active, and hydrolysis of GTP to GDP results in the inactivation of the protein. Conserved substitutions can be made to lock G-proteins in either the GTP- or GDP-bound form, thus causing constitutive activation or inactivation.

5. Mutations in portions of genes that encode regulatory domains of proteins: For example, many proteins, including kinases, contain regulatory domains that function as mechanisms to control the timing of activation. Mutations in these domains might result in the constitutive activation. Mutations that result in increased binding to regulatory proteins might result in constitutive inactivation.

Regulatory domains include short peptide sequences such as those required for nuclear import or export. Mutations in these sequences would result in constitutive

cytoplasmic or nuclear localization, respectively, which could either activate or inhibit signaling.

6. Mutations that result in proteins that are capable of binding to an appropriate signaling partner, but the complexes that form are inactive: For example, dimerization of proteins, either homodimers or heterodimers, often is required for signaling; in many instances, short protein sequences are sufficient to promote dimerization. Mutations in functional domains not required for dimerization might result in dominant inhibition; these proteins are capable of binding to and possibly sequestering other signaling molecules into inactive, or partially inactive, complexes.
7. Mutations that decrease or increase the targeting of proteins to the appropriate subcellular destination: Short peptide sequences often facilitate the targeting of proteins to specific subcellular locations. For example, short sequences are sufficient to be recognized and modified by fatty acylation, prenylation, or glycosyl-phosphatidylinositol modification. These modifications result in targeting of proteins to membranes. Membrane spanning peptide sequences also have been identified, as have targeting sequences for secretion. In addition, sequences have been identified that target proteins to subcellular locations such as the endoplasmic reticulum, mitochondria, peroxisome, vacuole, nucleus, and lysosome. Mutations that inhibit proper targeting might result in dominant inhibition; these proteins might be capable of binding to and possibly sequestering other signaling molecules from the appropriate subcellular location.
8. Mutations that create a new protein function. For example, a mutation in a protein kinase could result in altered substrate specificity, such that the mutated kinase can modulate the activity of pathways that it does not usually regulate.

In certain embodiments of the methods according to this aspect of the invention, the modulation is mediated by a peptide modulator of gene expression. The term "peptide" means a molecule comprised of a linear array of amino acid residues connected to each other in the linear array by peptide bonds. Such peptides according to the invention may include from about three to about 500 amino acids, and may further include secondary, tertiary or quaternary structures, as

well as intermolecular associations with other peptides or other non-peptide molecules. Such intermolecular associations may be through, without limitation, covalent bonding (*e.g.*, through disulfide linkages), or through chelation, electrostatic interactions, hydrophobic interactions, hydrogen bonding, ion-dipole interactions, dipole-dipole interactions, or any combination of the above. Peptides may be expressed in the cell or supplied exogenously. Preferably, they are provided on a scaffold to increase intracellular stability and to provide conformational constraint. A "scaffold" is a molecule, most frequently a small protein, from which a peptide is displayed; scaffolds are employed to optimize presentation, rigidity, conformational constraint, and potentially intracellular/extracellular localization. Preferred scaffolds include a catalytically inactive version of staphylococcal nuclease. Preferred peptides according to this aspect of the invention include, without limitation, those peptides disclosed in Norman *et al.*, Science 285: 591-595 (1999).

In certain embodiments of the methods according to this aspect of the invention, the peptide modulator is an activator of gene expression. An "activator of gene expression" is a molecule that causes transcription and/or translation and/or gene product maturation to exceed by at least two-fold, preferably at least five fold, and more preferably at least ten-fold, the level of such expression that would be present under similar growth conditions in the absence of the activator of expression of the gene. "Similar growth conditions" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the peptide modulator is an inhibitor of gene expression. An "inhibitor of gene expression" is a molecule that causes transcription and/or translation and/or gene product maturation to be reduced by at least two-fold, preferably at least five fold, and more preferably at least ten-fold, the level of such expression that would be present under similar growth conditions in the absence of the inhibitor of expression of the gene. "Similar growth conditions" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the modulation is mediated by a small molecule modulator of gene expression. In certain embodiments of the methods according to this aspect of the invention, the small molecule modulator is an activator of gene expression. The term "activator of gene expression" is as used before. In certain embodiments of the methods according to this aspect of the invention, the small molecule modulator is an inhibitor of gene expression. The term "inhibitor of gene

expression" is as used before. A "small molecule" is a compound with a preferable molecular weight below 1000 daltons.

In certain embodiments of the methods according to this aspect of the invention, the modulation is conditional expression of the gene. "Conditional expression" of a gene means expression under certain growth conditions, but not under others. Such growth conditions that may be varied include, without limitation, carbon source, nitrogen source, phosphate source, pH, temperature, partial oxygen pressure, the presence or absence of small molecules such as drugs, and the presence or absence of a solid substrate.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a transcription factor or the product that it encodes acts on a transcription factor. As used throughout this specification, the term "the gene acts on" means that the gene or its transcriptional, translational, or post-translationally modified product affects the function of its target (the word following the expression "the gene acts on"), preferably by increasing or decreasing transcription, increasing or decreasing translation, increasing or decreasing post-translational modification, increasing or decreasing protein stability, increasing or decreasing protein translocation, or increasing or decreasing protein function by interaction of the protein with another molecule. A "transcription factor" is a molecule that activates or inhibits transcription. The term "activates transcription" means to cause transcription to exceed by at least two-fold, preferably at least five fold, and more preferably at least ten-fold, the level of transcription that would be present under similar growth conditions in the absence of the transcription factor. The term "inhibits transcription" means to cause transcription to be reduced by at least two-fold, preferably at least five fold, and more preferably at least ten-fold, the level of such transcription that would be present under similar growth conditions in the absence of the transcription factor. Preferred transcription factors include, without limitation, transcription factors that modulate the expression of genes involved in the production or response to the small molecule cAMP (preferred examples include, without limitation, MGA1, MSN2, MSN4, SFL1, and SOK2); transcription factors that function downstream of mitogen-activated protein (MAP) kinase signaling pathways that regulate the yeast invasion response (preferred examples include, without limitation, MCM1, STE12, and TEC1); transcription factors that modulate the expression of genes involved in nitrogen regulation (preferred examples include, without

limitation, areA, GLN3, HMS1, HMS2, nreB, tamA, and UGA3); transcription factors that modulate the expression of genes involved in pH regulation in fungi (preferred examples include, without limitation pacC and RIM101); general transcription factors (preferred examples include, without limitation, SIN3, SNF2, SRB8, SRB9, SRB10, SRB11, SSN6, and TUP1); transcription factors that modulate the expression of genes involved in carbon metabolism (preferred examples include, without limitation, ADR1, CAT8, creA, facB, GCR1, GCR2, HAP4, MIG1, MIG2, MTH1, NRG1, OAF1, and SIP4); heme-dependent transcription factors (preferred examples include, without limitation, HAP1 and ROX1); transcription factors that modulate the expression of genes involved in the uptake of metals (preferred examples include, without limitation, AFT1, CUP9, MAC1, sreP, sreA, and ZAP1); transcription factors that modulate the expression of genes involved in cell-cycle regulation (preferred examples include, without limitation, SKN7, SWI4, and SWI6); transcription factors that modulate the expression of genes involved in invasion (preferred examples include, without limitation, ASH1, FLO8, GTS1, INV7, MSN1, MSS11, PHD1, and RRE1); transcription factors that modulate the expression of genes involved in amino acid biosynthesis or transport (preferred examples include, without limitation, GCN4, LEU3, LYS14, MET4, MET28, MET31, metR, PUT3, sconB, and UGA3); transcription factors that modulate the expression of genes involved in phosphate metabolism or transport (preferred examples include, without limitation, PHO2 and PHO4); transcription factors that modulate the expression of genes involved in nucleotide metabolism or transport (preferred examples include, without limitation, PPR1 and UaY); transcription factors that modulate the expression of genes involved in cell wall processes (preferred examples include, without limitation, ACE2, SWI4, and SWI6); transcription factors that modulate the expression of genes involved in sporulation (preferred examples include, without limitation, IME1 and IME4); transcription factors that modulate the expression of genes involved in phospholipid synthesis (preferred examples include, without limitation, INO2); transcription factors that modulate the expression of genes involved in aflatoxin biosynthesis (preferred examples include, without limitation, aflR); transcription factors that modulate the expression of genes involved in lovastatin biosynthesis (preferred examples include, without limitation, AAD34561 and lovE); and transcription factors that modulate the expression of genes involved in filamentous fungal development (preferred examples include, without limitation, abaA). The term "general transcription factors" means

components involved in the formation of preinitiation complexes at promoters that are regulated by RNA polymerase II. The term "invasion" means a process by which a fungus penetrates, digs, adheres to, or attaches to a substrate.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a transmembrane transporter or the product that it encodes acts on a transmembrane transporter. A "transmembrane transporter" is a molecule or complex of molecules that facilitates passage of another type of molecule from one side of a cellular membrane to the other side in an energy-dependent or energy-independent manner. "Facilitates passage" means that the number of molecules traversing the membrane is greater than it would have been in the absence of the transmembrane pump, preferably at least two-fold greater, more preferably at least ten-fold greater, even more preferably at least one hundred-fold greater, and most preferably at least one thousand-fold greater. Preferred classes of transmembrane transporters include, without limitation, proteins of the ATP-binding cassette superfamily, members of the Major Facilitator Superfamily (MFS), P-type ATPases, members of the mitochondrial carrier family (MCF) that include, without limitation, PET9; ion channels, permeases that include, without limitation, BAP2, HIP1, MEP1, and MEP2; and components that transport sugars, ions, or metals.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a kinase or the product that it encodes acts on a kinase. A "kinase" is a molecule that phosphorylates a protein, a lipid, a nucleic acid, a carbohydrate, or any other substrate that is capable of being phosphorylated. Preferred kinases include, without limitation, CDC28, ELM1, FUS3, GCN2, HOG1, HSL1, HXK2, KSS1, PBS2, PHO85, RIM15, STE7, SCH9, SNF1, STE11, STE20, TPK1, TPK2, and TPK3.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a G-protein or the product that it encodes acts on a G-protein. A "G-protein" is a guanyl-nucleotide binding protein. Preferred G-proteins include, without limitation CDC42, fadA, GPA1, GPA2, RAS1, and RAS2.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a cell surface receptor or the product that it encodes acts on a cell surface receptor. A "cell surface receptor" is a molecule that resides at the plasma membrane, binds an

extracellular signaling molecule, and transduces this signal to propagate a cellular response. Preferred cell surface receptors include, without limitation, G-protein coupled receptors. Preferred G-protein coupled receptors include, without limitation, GPR1.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a GTPase activating protein or the product that it encodes acts on a GTPase activating protein. A "GTPase activating protein" is a molecule that promotes the hydrolysis of GTP bound to a G-protein. GTP-activating proteins often negatively regulate the activity of G-proteins. Preferred GTPase activating proteins include, without limitation, RGS family members. "RGS family members" are regulators of G-protein signaling that act upon G-protein coupled receptors. Preferred RGS family members include, without limitation, FlbA, RGS2, and SST2.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a guanine nucleotide exchange factor or the product that it encodes acts on a guanine nucleotide exchange factor. A "guanine nucleotide exchange factor" is a molecule that catalyzes the dissociation of GDP from the inactive GTP-binding proteins; following dissociation, GTP can then bind and induce structural changes that activate G-protein signaling. Preferred guanine nucleotide exchange factors include, without limitation, CDC24 and CDC25.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a phosphatase or the product that it encodes acts on a phosphatase. A "phosphatase" is a molecule that dephosphorylates a protein, a lipid, a nucleic acid, a carbohydrate, or any other substrate that is capable of being dephosphorylated. Preferred phosphatases include, without limitation, CDC55 and PTC1.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a protease or the product that it encodes acts on a protease. A "protease" is a molecule that cleaves an amide bond in a peptide. "Peptide" is as used before. Preferred proteases include, without limitation, RIM13.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a cyclic nucleotide phosphodiesterase or the product that it encodes acts on a cyclic nucleotide phosphodiesterase. A "cyclic nucleotide phosphodiesterase" is a molecule catalyzes the hydrolysis of the 3' phosphate bond of a 3', 5' cyclic nucleotide to yield free 5'

nucleotide. Preferred examples of cyclic nucleotide phosphodiesterases include, without limitation, PDE2.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a bacterial protein toxin or the product that it encodes acts on a bacterial protein toxin. A "bacterial protein toxin" is protein produced by a bacterium, as part of the pathogenesis of the bacterial organism, to kill or impair the biological function of the host organism. Bacterial protein toxins exhibit a wide-variety of biochemical and enzymatic activities including those of adenylate cyclases, ADP-ribosyltransferases, phospholipases, and proteases. Expression of bacterial protein toxins in fungi could result in increased production of secondary metabolites. Preferred bacterial protein toxins include, without limitation, Anthrax toxin edema factor (EF; *Bacillus anthracis*), Anthrax toxin lethal factor (LF; *Bacillus anthracis*), adenylate cyclase toxin (*Bordetella pertussis*), Cholera enterotoxin (*Vibrio cholerae*), LT toxin (*Escherichia coli*), ST toxin (*E. coli*), Shiga toxin (*Shigella dysenteriae*), Perfringens enterotoxin (*Clostridium perfringens*), Botulinum toxin (*Clostridium botulinum*), Tetanus toxin (*Clostridium tetani*), Diphtheria toxin (*Corynebacterium diphtheriae*), Exotoxin A (*Pseudomonas aeruginosa*), Exoenzyme S (*P. aeruginosa*), Pertussis toxin (*Bordetella pertussis*), alpha and epsilon toxins (*C. perfringens*), lethal toxin (LT; *Clostridium sordellii*), toxins A and B (*Clostridium dificile*), and phospholipase C (*Clostridium bifermentans*).

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes an importin protein or the product that it encodes acts on a importin protein. An "importin" protein is a molecule that functions in the translocation of proteins from the nucleus to the cytosol or from the cytosol from the nucleus. Preferred examples of importin proteins include, without limitation, MSN5.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes an RNA-binding protein or the product that it encodes acts on an RNA-binding protein. Preferred examples of RNA-binding proteins include, without limitation, DHH1 and WHI3.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a component of a SCF complex or the product that it encodes acts on a component of a SCF complex. A "component of a SCF complex" is a molecule in a multi-protein aggregate

that targets various substrates involved in the G1 to S phase cell cycle transition for ubiquitin-dependent degradation. Preferred examples of components of a SCF complex include, without limitation, GRR1.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a biosynthetic enzyme or the product that it encodes acts on a biosynthetic enzyme. The term "biosynthetic enzyme" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-bacterial. An "anti-bacterial" is a molecule that has cytocidal or cytostatic activity against some or all bacteria. Preferred anti-bacterials include, without limitation, B-lactams. Preferred B-lactams include, without limitation, penicillins and cephalosporins. Preferred penicillins and biosynthetic intermediates include, without limitation, isopenicillin N, 6-aminopenicillanic acid (6-APA), penicillin G, penicillin N, and penicillin V. Preferred cephalosporins and biosynthetic intermediates include, without limitation, deacetoxycephalosporin V (DAOC V), deacetoxycephalosporin C (DAOC), deacetylcephalosporin C (DAC), 7-aminodeacetoxycephalosporanic acid (7-ADCA), cephalosporin C, 7- B -(5-carboxy-5-oxopentanamido)-cephalosporanic acid (keto-AD-7ACA), 7- B -(4-carboxybutanamido)-cephalosporanic acid (GL-7ACA), and 7-aminocephalosporanic acid (7ACA).

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-hypercholesterolemic. An "anti-hypercholesterolemic" is a drug administered to a patient diagnosed with elevated cholesterol levels, for the purpose of lowering the cholesterol levels. Preferred anti-hypercholesterolemics include, without limitation, lovastatin, mevastatin, simvastatin, and pravastatin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an immunosuppressant. An "immunosuppressant" is a molecule that reduces or eliminates an immune response in a host when the host is challenged with an immunogenic molecule, including immunogenic molecules present on transplanted organs, tissues or cells. Preferred immunosuppressants include, without limitation, members of the

cyclosporin family and beauverolide L. Preferred cyclosporins include, without limitation, cyclosporin A and cyclosporin C.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an ergot alkaloid. An "ergot alkaloid" is a member of a large family of alkaloid compounds that are most often produced in the sclerotia of fungi of the genus Claviceps. An "alkaloid" is a small molecule that contains nitrogen and has basic pH characteristics. The classes of ergot alkaloids include clavine alkaloids, lysergic acids, lysergic acid amides, and ergot peptide alkaloids. Preferred ergot alkaloids include, without limitation, ergotamine, ergosine, ergocristine, ergocryptine, ergocornine, ergotaminine, ergosinine, ergocristinine, ergocryptinine, ergocorninine, ergonovine, ergometrinine, and ergoclavine.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an inhibitor of angiogenesis. An "angiogenesis inhibitor" is a molecule that decreases or prevents the formation of new blood vessels. Angiogenesis inhibitors have proven effective in the treatment of several human diseases including, without limitation, cancer, rheumatoid arthritis, and diabetic retinopathy. Preferred inhibitors of angiogenesis include, without limitation, fumagillin and ovalicin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a glucan synthase inhibitor. A "glucan synthase inhibitor" is a molecule that decreases or inhibits the production of 1,3-B-D-glucan, a structural polymer of fungal cell walls. Glucan synthase inhibitors are a class of antifungal agents. Preferred glucan synthase inhibitors include, without limitation, echinocandin B, pneumocandin B, aculeacin A, and papulacandin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a member of the gliotoxin family of compounds. The "gliotoxin family of compounds" are related molecules of the epipolythiodioxopiperazine class. Gliotoxins display diverse biological activities, including, without limitation, antimicrobial, antifungal, antiviral, and immunomodulating activities. Preferred members of the "gliotoxin family of compounds" include, without limitation, gliotoxin and aspirochlorine.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a fungal toxin. A "fungal toxin" is a compound that causes a

pathological condition in a host, either plant or animal. Fungal toxins could be mycotoxins present in food products, toxins produced by phytopathogens, toxins from poisonous mushrooms, or toxins produced by zoopathogens. Preferred fungal toxins include, without limitation, aflatoxins, patulin, zearalenone, cytochalasin, griseofulvin, ergochrome, cercosporin, marticin, xanthocillin, coumarins, tricothecenes, fusidanes, sesterpenes, amatoxins, malformin A, phallotoxins, pentoxin, HC toxin, psilocybin, bufotenine, lysergic acid, sporodesmin, pulcheriminic acid, sordarins, fumonisins, ochratoxin A, and fusaric acid.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a modulator of cell surface receptor signaling. The term "cell surface receptor" is as used before. Modulators of cell surface receptor signaling might function by one of several mechanisms including, without limitation, acting as agonists or antagonists, sequestering a molecule that interacts with a receptor such as a ligand, or stabilizing the interaction of a receptor and molecule with which it interacts. Preferred modulators of cell surface signaling include, without limitation, the insulin receptor agonist L-783,281 and the cholecystokinin receptor antagonist asperlicin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a plant growth regulator. A "plant growth regulator" is a molecule that controls growth and development of a plant by affecting processes that include, without limitation, division, elongation, and differentiation of cells. Preferred plant growth regulators include, without limitation, cytokinin, auxin, gibberellin, abscisic acid, and ethylene.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a pigment. A "pigment" is a substance that imparts a characteristic color. Preferred pigments include, without limitation, melanins and carotenoids.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an insecticide. An "insecticide" is a molecule that is toxic to insects. Preferred insecticides include, without limitation, nodulisporic acid.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-neoplastic compound. An "anti-neoplastic" compound is a molecule that prevents or reduces tumor formation. Preferred anti-neoplastic compounds include, without limitation, taxol (paclitaxel) and related taxoids.

In certain embodiments of the methods according to this aspect of the invention, the gene is not AFL1, BEM2, CDC25, DHH1, HOG1, INV11, INV13, INV5, INV7, INV9, IRA1, MCM1, MEP2, MGA1, MSN1, MSN5, MSS11, PET9, PHO23, PTC1, RIM101, RIM13, RIM15, RIM9, SFL1, SNF8, SRB11, SSD1, STP22, SWI4, TPK2, TPK3, VPS28, VPS36, or YPR1. Each of these genes is as described in PCT Publication No. WO99/25865A1

In certain embodiments of the methods according to this aspect of the invention, the gene is selected from the group consisting of AAD34561, abaA, ACE2, ADR1, AFL1, aflR, AFT1, amyR, areA, ASH1, BAP2, BCY1, CAT8, CDC24, CDC25, CDC28, CDC42, CDC55, CLB2, creA, CTS1, CUP9, CYR1, DFG16, DHH1, DPH3, ELM1, facB, FLO1, FLO11, FLO8, FUS3, GCN2, GCN4, GCR1, GCR2, GLN3, GPA1, GPA2, GPR1, GRR1, GTS1, HAP1, HAP4, HIP1, HMS1, HMS2, HOG1, HSL1, HXK2, IME1, IME4, INO2, INV11, INV13, INV16, INV5, INV7, INV9, KSS1, LEU3, lovE, LYS14, MAC1, MCM1, MEP1, MEP2, MET28, MET31, MET4, metR, MGA1, MIG1, MIG2, MSN1, MSN2, MSN4, MSN5, MSS11, MTH1, NPR1, nreB, NRG1, OAF1, pacC, PBS2, PDE2, PET9, PHD1, PHO2, PHO4, PHO85, pkaR, PPR1, PTC1, PUT3, RAS1, RAS2, RGS2, RIM101, RIM13, RIM15, RIM9, ROX1, RRE1, SCH9, sconB, SFL1, SHO1, SHR3, SIN3, SIP4, SKN7, SNF1, SNF2, SNF7, SNF8, SOK2, SRB10, SRB11, SRB8, SRB9, sreA, sreP, SRV2, SSD1, SSN6, SST2, STE11, STE12, STE20, STE50, STE7, STP22, SWI4, SWI6, tamA, TEC1, TPK1, TPK2, TPK3, TUP1, UaY, UGA3, URE2, VPS28, VPS36, WHI3, YMR077c, YNL255c, YPR1, ZAP1, genes encoding bacterial protein toxins, and any fungal homologs of the aforementioned genes. A "fungal homolog" of a gene is a gene encoding a gene product that is capable of performing at least a portion of the function of the product encoded by the reference gene, and is substantially identical to the reference gene and/or the encoded product. "Substantially identical" means a polypeptide or nucleic acid exhibiting at least 25%, preferably 50%, more preferably 80%, and most preferably 90%, or even 95% identity to a reference amino acid sequence or nucleic acid sequence. For polypeptides, the length of comparison sequences will generally be at least 16 amino acids, preferably at least 20 amino acids, more preferably at least 25 amino acids, and most preferably 35 amino acids or greater. For nucleic acids, the length of comparison sequences will generally be at least 50 nucleotides,

preferably at least 60 nucleotides, more preferably at least 75 nucleotides, and most preferably 110 nucleotides or greater.

Sequence identity is typically measured using sequence analysis software (for example, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison WI 53705, BLAST, BEAUTY, or PILEUP/PRETTYBOX programs). Such software matches identical or similar sequences by assigning degrees of homology to various substitutions, deletions, and/or other modifications. Conservative substitutions typically include substitutions within the following group: glycine, alanine, valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

In certain embodiments of the methods according to this aspect of the invention, the methods further comprise purifying the secondary metabolite from a culture of the fungus. "Purifying" means obtaining the secondary metabolite in substantially pure form. "Substantially pure" means comprising at least 90 %, more preferably at least 95 %, and most preferably at least 99 %, of the purified composition on a weight basis.

In a second aspect, the invention provides methods for improving production of a secondary metabolite by a fungus by increasing productivity of the secondary metabolite in the fungus, the methods comprising modulating the expression of a gene involved in regulation of secondary metabolite production in a manner that improves the productivity of the secondary metabolite. "Increasing productivity" means to increase the quotient for the equation concentration of secondary metabolite divided by the product of time of fermentor run, fermentation volume, and grams of dry cell weight of biomass (Productivity= concentration metabolite/ (time*volume*gDCW)). Significant advantages that might result from increasing productivity include, without limitation, a decrease in fermentor run time, a decrease in the size of fermentor required for production of equivalent amounts of secondary metabolite, or a decrease in the biomass required for production, which translates into decrease waste that must be handled in downstream processing. Preferably, such increased productivity is by at least ten percent, more preferably at least 50 percent, and most preferably at least two-fold.

Preferably, for this aspect of the invention, when the secondary metabolite is isopenicillin N, then the modulation is not mediated by the transcription factor CPCR1; when the secondary metabolite is sterigmatocystin, then the modulation is not through AflR, FadA, or FluG; when the secondary metabolite is aflatoxin, then the modulation is not through AflR; when the secondary metabolite is penicillin and the fungus is Aspergillus, then the modulation is not through mutations that result in expression of truncated forms of PacC or constitutively active forms of FadA; when the gene involved in regulation of secondary metabolite production is from *Saccharomyces cerevisiae*, then the modulation is not through decreased activity or expression of BEM2, HOG1, IRA1, RIM15, SFL1, SRB11, SSD1, SWI4, TPK3 or though increased activity or expression of AFL1, CDC25, DHH1, HAP4, INV11, INV13, INV5, INV7, INV9, MCM1, MEP2, MGA1, MSN1, MSN5, MSS11, PET9, PHO23, PTC1, RIM101, RIM13, RIM9, SNF8, STP22, TPK2, VPS28, VPS36, or YPR1.

In certain embodiments of the methods according to this aspect of the invention, the modulation is overexpression of the gene. "Overexpression of the gene" is as used before. Preferred genes according to this aspect of the invention include, without limitation, AAD34561, abaA, ACE2, ADR1, AFL1, aflR, AFT1, amyR, areA, ASH1, BAP2, BCY1, CAT8, CDC24, CDC25, CDC28, CDC42, CDC55, CLB2, creA, CTS1, CUP9, CYR1, DFG16, DHH1, DPH3, ELM1, facB, FLO1, FLO11, FLO8, FUS3, GCN2, GCN4, GCR1, GCR2, GLN3, GPA1, GPA2, GPR1, GRR1, GTS1, HAP1, HAP4, HIP1, HMS1, HMS2, HOG1, HSL1, HXK2, IME1, IME4, INO2, INV11, INV13, INV16, INV5, INV7, INV9, KSS1, LEU3, lovE, LYS14, MAC1, MCM1, MEP1, MEP2, MET28, MET31, MET4, metR, MGA1, MIG1, MIG2, MSN1, MSN2, MSN4, MSN5, MSS11, MTH1, NPR1, nreB, NRG1, OAF1, pacC, PBS2, PDE2, PET9, PHD1, PHO2, PHO4, PHO85, pkaR, PPR1, PTC1, PUT3, RAS1, RAS2, RGS2, RIM101, RIM13, RIM15, RIM9, ROX1, RRE1, SCH9, sconB, SFL1, SHO1, SHR3, SIN3, SIP4, SKN7, SNF1, SNF2, SNF7, SNF8, SOK2, SRB10, SRB11, SRB8, SRB9, sreA, sreP, SRV2, SSD1, SSN6, SST2, STE11, STE12, STE20, STE50, STE7, STP22, SWI4, SWI6, tamA, TEC1, TPK1, TPK2, TPK3, TUP1, UaY, UGA3, URE2, VPS28, VPS36, WHI3, YMR077c, YNL255c, YPR1, ZAP1, genes encoding bacterial protein toxins, and any fungal homologs of the aforementioned genes.

In certain embodiments of the methods according to this aspect of the invention, the modulation is expression of a dominant mutation of the gene. The term "dominant mutation" is

as used before. Preferred dominant mutations according to this aspect of the invention are as used before.

In certain embodiments of the methods according to this aspect of the invention, the modulation is mediated by a peptide modulator of gene expression. The term "peptide" is as used before. Peptides may be expressed in the cell or supplied exogenously. Preferably, they are provided on a scaffold to increase intracellular stability and to provide conformational constraint. Preferred peptides according to this aspect of the invention include those discussed earlier.

In certain embodiments of the methods according to this aspect of the invention, the peptide modulator is an activator of gene expression. The term "activator of gene expression" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the peptide modulator is an inhibitor of gene expression. The term "inhibitor of gene expression" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the modulation is mediated by a small molecule modulator of gene expression. In certain embodiments of the methods according to this aspect of the invention, the small molecule modulator is an activator of gene expression. The term "activator of gene expression" is as used before. In certain embodiments of the methods according to this aspect of the invention, the small molecule modulator is an inhibitor of gene expression. The term "inhibitor of gene expression" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the modulation is conditional expression of the gene. The term "conditional expression" of a gene is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a transcription factor or the product that it encodes acts on a transcription factor. The term "the gene acts on" is as used before. The term "transcription factor" is as used before. Preferred transcription factors include, without limitation, transcription factors that modulate the expression of genes involved in the production or response to the small molecule cAMP (preferred examples include, without limitation, MGA1, MSN2, MSN4, SFL1, and SOK2); transcription factors that function downstream of mitogen-activated protein (MAP) kinase

signaling pathways that regulate the yeast invasion response (preferred examples include, without limitation, MCM1, STE12, and TEC1); transcription factors that modulate the expression of genes involved in nitrogen regulation (preferred examples include, without limitation, areA, GLN3, HMS1, HMS2, nreB, tamA, and UGA3); transcription factors that modulate the expression of genes involved in pH regulation in fungi (preferred examples include, without limitation pacC and RIM101); general transcription factors (preferred examples include, without limitation, SIN3, SNF2, SRB8, SRB9, SRB10, SRB11, SSN6, and TUP1); transcription factors that modulate the expression of genes involved in carbon metabolism (preferred examples include, without limitation, ADR1, CAT8, creA, facB, GCR1, GCR2, HAP4, MIG1, MIG2, MTH1, NRG1, OAF1, and SIP4); heme-dependent transcription factors (preferred examples include, without limitation, HAP1 and ROX1); transcription factors that modulate the expression of genes involved in the uptake of metals (preferred examples include, without limitation, AFT1, CUP9, MAC1, sreP, sreA, and ZAP1); transcription factors that modulate the expression of genes involved in cell-cycle regulation (preferred examples include, without limitation, SKN7, SWI4, and SWI6); transcription factors that modulate the expression of genes involved in invasion (preferred examples include, without limitation, ASH1, FLO8, GTS1, INV7, MSN1, MSS11, PHD1, and RRE1); transcription factors that modulate the expression of genes involved in amino acid biosynthesis or transport (preferred examples include, without limitation, GCN4, LEU3, LYS14, MET4, MET28, MET31, metR, PUT3, sconB, and UGA3); transcription factors that modulate the expression of genes involved in phosphate metabolism or transport (preferred examples include, without limitation, PHO2 and PHO4); transcription factors that modulate the expression of genes involved in nucleotide metabolism or transport (preferred examples include, without limitation, PPR1 and UaY); transcription factors that modulate the expression of genes involved in cell wall processes (preferred examples include, without limitation, ACE2, SWI4, and SWI6); transcription factors that modulate the expression of genes involved in sporulation (preferred examples include, without limitation, IME1 and IME4); transcription factors that modulate the expression of genes involved in phospholipid synthesis (preferred examples include, without limitation, INO2); transcription factors that modulate the expression of genes involved in aflatoxin biosynthesis (preferred examples include, without limitation, aflR); transcription factors that modulate the expression of genes involved in lovastatin biosynthesis

(preferred examples include, without limitation, AAD34561 and lovE); and transcription factors that modulate the expression of genes involved in filamentous fungal development (preferred examples include, without limitation, abaA). The term "general transcription factors" is as used before. The term "invasion" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a transmembrane transporter or the product that it encodes acts on a transmembrane transporter. The term "transmembrane transporter" is as used before. Preferred classes of transmembrane transporters include, without limitation, proteins of the ATP-binding cassette superfamily, members of the Major Facilitator Superfamily (MFS), P-type ATPases, members of the mitochondrial carrier family (MCF) that include, without limitation, PET9; ion channels, permeases that include, without limitation, BAP2, HIP1, MEP1, and MEP2; and components that transport sugars, ions, or metals.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a kinase or the product that it encodes acts on a kinase. The term "kinase" is as used before. Preferred kinases include, without limitation, CDC28, ELM1, FUS3, GCN2, HOG1, HSL1, HXK2, KSS1, PBS2, PHO85, RIM15, STE7, SCH9, SNF1, STE11, STE20, TPK1, TPK2, and TPK3.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a G-protein or the product that it encodes acts on a G-protein. The term "G-protein" is as used before. Preferred G-proteins include, without limitation CDC42, fadA, GPA1, GPA2, RAS1, and RAS2.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a cell surface receptor or the product that it encodes acts on a cell surface receptor. The term "cell surface receptor" is as used before. Preferred cell surface receptors include, without limitation, G-protein coupled receptors. Preferred G-protein coupled receptors include, without limitation, GPR1.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a GTPase activating protein or the product that it encodes acts on a GTPase activating protein. The term "GTPase activating protein" is as used before. Preferred GTPase activating proteins include, without limitation, RGS family members. "RGS family members"

are regulators of G-protein signaling that act upon G-protein coupled receptors. Preferred RGS family members include, without limitation, FlbA, RGS2, and SST2.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a guanine nucleotide exchange factor or the product that it encodes acts on a guanine nucleotide exchange factor. The term "guanine nucleotide exchange factor" is as used before. Preferred guanine nucleotide exchange factors include, without limitation, CDC24 and CDC25.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a phosphatase or the product that it encodes acts on a phosphatase. The term "phosphatase" is as used before. Preferred phosphatases include, without limitation, CDC55 and PTC1.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a protease or the product that it encodes acts on a protease. The term "protease" is as used before. Preferred proteases include, without limitation, RIM13.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a cyclic nucleotide phosphodiesterase or the product that it encodes acts on a cyclic nucleotide phosphodiesterase. The term "cyclic nucleotide phosphodiesterase" is as used before. Preferred examples of cyclic nucleotide phosphodiesterases include, without limitation, PDE2.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a bacterial protein toxin or the product that it encodes acts on a bacterial protein toxin. The term "bacterial protein toxin" is as used before. Preferred bacterial protein toxins include, without limitation, Anthrax toxin edema factor (EF; *Bacillus anthracis*), Anthrax toxin lethal factor (LF; *Bacillus anthracis*), adenylate cyclase toxin (*Bordetella pertussis*), Cholera enterotoxin (*Vibrio cholerae*), LT toxin (*Escherichia coli*), ST toxin (*E. coli*), Shiga toxin (*Shigella dysenteriae*), Perfringens enterotoxin (*Clostridium perfringens*), Botulinum toxin (*Clostridium botulinum*), Tetanus toxin (*Clostridium tetani*), Diphtheria toxin (*Corynebacterium diphtheriae*), Exotoxin A (*Pseudomonas aeruginosa*), Exoenzyme S (*P. aeruginosa*), Pertussis toxin (*Bordetella pertussis*), alpha and epsilon toxins (*C. perfringens*), lethal toxin (LT;

Clostridium sordellii), toxins A and B (*Clostridium dificile*), and phospholipase C (*Clostridium bifermentans*).

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes an importin protein or the product that it encodes acts on a importin protein. The term "importin" protein is as used before. Preferred examples of importin proteins include, without limitation, MSN5.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes an RNA-binding protein or the product that it encodes acts on an RNA-binding protein. Preferred examples of RNA-binding proteins include, without limitation, DHH1 and WHI3.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a component of a SCF complex or the product that it encodes acts on a component of a SCF complex. The term "component of a SCF complex" is as used before. Preferred examples of components of a SCF complex include, without limitation, GRR1.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a biosynthetic enzyme or the product that it encodes acts on a biosynthetic enzyme. The term "biosynthetic enzyme" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-bacterial. The term "anti-bacterial" is as used before. Preferred anti-bacterials include, without limitation, B-lactams. Preferred B-lactams include, without limitation, penicillins and cephalosporins. Preferred penicillins and biosynthetic intermediates include, without limitation, isopenicillin N, 6-aminopenicillanic acid (6-APA), penicillin G, penicillin N, and penicillin V. Preferred cephalosporins and biosynthetic intermediates include, without limitation, deacetoxycephalosporin V (DAOC V), deacetoxycephalosporin C (DAOC), deacetylcephalosporin C (DAC), 7-aminodeacetoxycephalosporanic acid (7-ADCA), cephalosporin C, 7- B -(5-carboxy-5-oxopentanamido)-cephalosporanic acid (keto-AD-7ACA), 7- B -(4-carboxybutanamido)-cephalosporanic acid (GL-7ACA), and 7-aminocephalosporanic acid (7ACA).

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-hypercholesterolemic. An "anti-hypercholesterolemic" is as used

before. Preferred anti-hypercholesterolemics include, without limitation, lovastatin, mevastatin, simvastatin, and pravastatin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an immunosuppressant. An "immunosuppressant" is as used before. Preferred immunosuppressants include, without limitation, members of the cyclosporin family and beauverolide L. Preferred cyclosporins include, without limitation, cyclosporin A and cyclosporin C.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an ergot alkaloid. The term "ergot alkaloid" is as used before. Preferred classes of ergot alkaloids include clavine alkaloids, lysergic acids, lysergic acid amides, and ergot peptide alkaloids. Preferred ergot alkaloids include, without limitation, ergotamine, ergosine, ergocristine, ergocryptine, ergocornine, ergotaminine, ergosinine, ergocristinine, ergocryptinine, ergocorninine, ergonovine, ergometrinine, and ergoclavine.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an inhibitor of angiogenesis. The term "inhibitor of angiogenesis" is as used before. Preferred inhibitors of angiogenesis include, without limitation, fumagillin and ovalicin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a glucan synthase inhibitor. The term "glucan synthase inhibitor" is as used before. Preferred glucan synthase inhibitors include, without limitation, echinocandin B, pneumocandin B, aculeacin A, and papulacandin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a member of the gliotoxin family of compounds. The term "gliotoxin family of compounds" is as used before. Preferred members of the "gliotoxin family of compounds" include, without limitation, gliotoxin and aspirochlorine.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a fungal toxin. The term "fungal toxin" is as used before. Preferred fungal toxins include, without limitation, aflatoxins, patulin, zearalenone, cytochalasin, griseofulvin, ergochrome, cercosporin, marticin, xanthocillin, coumarins, tricothecenes, fusidanes, sesterpenes, amatoxins, malformin A, phallotoxins, pentoxin, HC toxin, psilocybin,

bufotenine, lysergic acid, sporodesmin, pulcheriminic acid, sordarins, fumonisins, ochratoxin A, and fusaric acid.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a modulator of cell surface receptor signaling. The term "cell surface receptor" is as used before. Preferred modulators of cell surface signaling include, without limitation, the insulin receptor agonist L-783,281 and the cholecystokinin receptor antagonist asperlicin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a plant growth regulator. The term "plant growth regulator" is as used before. Preferred plant growth regulators include, without limitation, cytokinin, auxin, gibberellin, abscisic acid, and ethylene.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a pigment. The term "pigment" is as defined before. Preferred pigments include, without limitation, melanins and carotenoids.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an insecticide. The term "insecticide" is as used before. Preferred insecticides include, without limitation, nodulisporic acid.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-neoplastic compound. The term "anti-neoplastic" compound is as used before. Preferred anti-neoplastic compounds include, without limitation, taxol (paclitaxel) and related taxoids.

In certain embodiments of the methods according to this aspect of the invention, the gene is selected from the group consisting of AAD34561, abaA, ACE2, ADR1, AFL1, aflR, AFT1, amyR, areA, ASH1, BAP2, BCY1, CAT8, CDC24, CDC25, CDC28, CDC42, CDC55, CLB2, creA, CTS1, CUP9, CYR1, DFG16, DHH1, DPH3, ELM1, facB, FLO1, FLO11, FLO8, FUS3, GCN2, GCN4, GCR1, GCR2, GLN3, GPA1, GPA2, GPR1, GRR1, GTS1, HAP1, HAP4, HIP1, HMS1, HMS2, HOG1, HSL1, HXK2, IME1, IME4, INO2, INV11, INV13, INV16, INV5, INV7, INV9, KSS1, LEU3, lovE, LYS14, MAC1, MCM1, MEP1, MEP2, MET28, MET31, MET4, metR, MGA1, MIG1, MIG2, MSN1, MSN2, MSN4, MSN5, MSS11, MTH1, NPR1, nreB,

NRG1, OAF1, pacC, PBS2, PDE2, PET9, PHD1, PHO2, PHO4, PHO85, pkaR, PPR1, PTC1, PUT3, RAS1, RAS2, RGS2, RIM101, RIM13, RIM15, RIM9, ROX1, RRE1, SCH9, SFL1, SHO1, SHR3, SIN3, SIP4, SKN7, SNF1, SNF2, SNF7, SNF8, sconB, SOK2, SRB10, SRB11, SRB8, SRB9, sreA, sreP, SRV2, SSD1, SSN6, SST2, STE11, STE12, STE20, STE50, STE7, STP22, SWI4, SWI6, tamA, TEC1, TPK1, TPK2, TPK3, TUP1, UaY, UGA3, URE2, VPS28, VPS36, WHI3, YMR077c, YNL255c, YPR1, ZAP1, genes encoding bacterial protein toxins, and any fungal homologs of the aforementioned genes. The term "fungal homolog" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the methods further comprise purifying the secondary metabolite from a culture of the fungus. The term "purifying" is as used before.

In a third aspect, the invention provides methods for improving production of a secondary metabolite in a fungus by increasing efflux or excretion of the secondary metabolite, the method comprising modulating the expression of a gene involved in regulation of secondary metabolite production in a manner that increases efflux or excretion of the secondary metabolite. "Increasing efflux or excretion of the secondary metabolite" means that a greater quantity of the secondary metabolite passes from the inside of the fungal cells to the outside of the fungal cell per unit time in the absence of lysis of the fungal cells. "Outside of the fungal cell" is defined as being no longer contained wholly within the lipid bilayer of the cell and/or extractable from the cell with methods which do not release a majority of intracellular contents.

In certain embodiments of the methods according to this aspect of the invention, the modulation is overexpression of the gene. "Overexpression of the gene" is as used before. Preferred genes according to this aspect of the invention include, without limitation, AAD34558, AAD34561, AAD34564, ATR1, ERG6, FCR1, GCN4, lovE, MDR1, PDR1, PDR3, PDR5, PDR10, PDR13, SNQ2, TRI12, and YAP1.

In certain embodiments of the methods according to this aspect of the invention, the modulation is expression of a dominant mutation of the gene. The term "dominant mutation" is as used before. Preferred dominant mutations according to this aspect of the invention are as used before.

In certain embodiments of the methods according to this aspect of the invention, the modulation is mediated by a peptide modulator of gene expression. The term "peptide" is as used before. Peptides may be expressed in the cell or supplied exogenously. Preferably, they are provided on a scaffold to increase intracellular stability and to provide conformational constraint. Preferred peptides according to this aspect of the invention include those discussed earlier.

In certain embodiments of the methods according to this aspect of the invention, the peptide modulator is an activator of gene expression. The term "activator of gene expression" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the peptide modulator is an inhibitor of gene expression. The term "inhibitor of gene expression" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the modulation is mediated by a small molecule modulator of gene expression. In certain embodiments of the methods according to this aspect of the invention, the small molecule modulator is an activator of gene expression. The term "activator of gene expression" is as used before. In certain embodiments of the methods according to this aspect of the invention, the small molecule modulator is an inhibitor of gene expression. The term "inhibitor of gene expression" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the modulation is conditional expression of the gene. The term "conditional expression" of a gene is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a transcription factor or the product that it encodes acts on a transcription factor. The term "transcription factor" is as used before. Preferred transcription factors include, without limitation, AAD34561, FCR1, GCN4, lovE, PDR1, PDR3, and YAP1.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a transmembrane transporter or the product that it encodes acts on a transmembrane transporter. The term "transmembrane transporter" is as used before. Preferred transmembrane transporters include, without limitation, AAD34558, AAD34564, ATR1, MDR1, PDR5, PDR10, SNQ2, and TRI12.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a kinase or the product that it encodes acts on a kinase. A "kinase" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a G-protein or the product that it encodes acts on a G-protein. A "G-protein" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a cell surface receptor or the product that it encodes acts on a cell surface receptor. A "cell surface receptor" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a GTPase activating protein or the product that it encodes acts on a GTPase activating protein. A "GTPase activating protein" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a guanine nucleotide exchange factor or the product that it encodes acts on a guanine nucleotide exchange factor. A "guanine nucleotide exchange factor" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a phosphatase or the product that it encodes acts on a phosphatase. A "phosphatase" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a protease or the product that it encodes acts on a protease. A "protease" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a cyclic nucleotide phosphodiesterase or the product that it encodes acts on a cyclic nucleotide phosphodiesterase. A "cyclic nucleotide phosphodiesterase" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a bacterial protein toxin or the product that it encodes acts on a bacterial protein toxin. A "bacterial protein toxin" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes an importin protein or the product that it encodes acts on a importin protein. An "importin" protein is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes an RNA-binding protein or the product that it encodes acts on an RNA-binding protein.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a component of a SCF complex or the product that it encodes acts on a component of a SCF complex. A "component of a SCF complex" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a biosynthetic enzyme or the product that it encodes acts on a biosynthetic enzyme. The term "biosynthetic enzyme" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-bacterial. The term "anti-bacterial" is as used before. Preferred anti-bacterials include, without limitation, B-lactams. Preferred B-lactams include, without limitation, penicillins and cephalosporins. Preferred penicillins and biosynthetic intermediates include, without limitation, isopenicillin N, 6-aminopenicillanic acid (6-APA), penicillin G, penicillin N, and penicillin V. Preferred cephalosporins and biosynthetic intermediates include, without limitation, deacetoxycephalosporin V (DAOC V), deacetoxycephalosporin C (DAOC), deacetylcephalosporin C (DAC), 7-aminodeacetoxycephalosporanic acid (7-ADCA), cephalosporin C, 7- B -(5-carboxy-5-oxopentanamido)-cephalosporanic acid (keto-AD-7ACA), 7- B -(4-carboxybutanamido)-cephalosporanic acid (GL-7ACA), and 7-aminocephalosporanic acid (7ACA).

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-hypercholesterolemic. An "anti-hypercholesterolemic" is as used before. Preferred anti-hypercholesterolemics include, without limitation, lovastatin, mevastatin, simvastatin, and pravastatin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an immunosuppressant. An "immunosuppressant" is as used before. Preferred immunosuppressants include, without limitation, members of the cyclosporin family and beauverolide L. Preferred cyclosporins include, without limitation, cyclosporin A and cyclosporin C.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an ergot alkaloid. The term "ergot alkaloid" is as used before. Preferred

classes of ergot alkaloids include clavine alkaloids, lysergic acids, lysergic acid amides, and ergot peptide alkaloids. Preferred ergot alkaloids include, without limitation, ergotamine, ergosine, ergocristine, ergocryptine, ergocornine, ergotaminine, ergosinine, ergocristinine, ergocryptinine, ergocorninine, ergonovine, ergometrinine, and ergoclavine.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an inhibitor of angiogenesis. The term "inhibitor of angiogenesis" is as used before. Preferred inhibitors of angiogenesis include, without limitation, fumagillin and ovalicin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a glucan synthase inhibitor. The term "glucan synthase inhibitor" is as used before. Preferred glucan synthase inhibitors include, without limitation, echinocandin B, pneumocandin B, aculeacin A, and papulacandin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a member of the gliotoxin family of compounds. The term "gliotoxin family of compounds" is as used before. Preferred members of the "gliotoxin family of compounds" include, without limitation, gliotoxin and aspirochlorine.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a fungal toxin. The term "fungal toxin" is as used before. Preferred fungal toxins include, without limitation, aflatoxins, patulin, zearalenone, cytochalasin, griseofulvin, ergochrome, cercosporin, marticin, xanthocillin, coumarins, tricothecenes, fusidanes, sesterpenes, amatoxins, malformin A, phallotoxins, pentoxin, HC toxin, psilocybin, bufotenine, lysergic acid, sporodesmin, pulcheriminic acid, sordarins, fumonisins, ochratoxin A, and fusaric acid.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a modulator of cell surface receptor signaling. The term "cell surface receptor" is as used before. Preferred modulators of cell surface signaling include, without limitation, the insulin receptor agonist L-783,281 and the cholecystokinin receptor antagonist asperlicin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a plant growth regulator. The term "plant growth regulator" is as used

before. Preferred plant growth regulators include, without limitation, cytokinin, auxin, gibberellin, abscisic acid, and ethylene.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a pigment. The term "pigment" is as defined before. Preferred pigments include, without limitation, melanins and carotenoids.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an insecticide. The term "insecticide" is as used before. Preferred insecticides include, without limitation, nodulisporic acid.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-neoplastic compound. The term "anti-neoplastic" compound is as used before. Preferred anti-neoplastic compounds include, without limitation, taxol (paclitaxel) and related taxoids.

In certain embodiments of the methods according to this aspect of the invention, the gene is selected from the group consisting of AAD34558, AAD34561, AAD34564, ATR1, ERG6, FCR1, GCN4, lovE, MDR1, PDR1, PDR3, PDR5, PDR10, PDR13, SNQ2, TRI12, YAP1, and any fungal homologs of the aforementioned genes. The term "fungal homolog" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the methods further comprise purifying the secondary metabolite from a culture of the fungus. The term "purifying" is as used before.

In a fourth aspect, the invention provides methods for improving production of a secondary metabolite in a fungus by decreasing production of side products or non-desired secondary metabolites, the method comprising modulating the expression of a gene involved in regulation of secondary metabolite production in a manner that decreases production of side products or non-desired secondary metabolites.

In certain embodiments of the methods according to this aspect of the invention, the modulation is overexpression of the gene. "Overexpression of the gene" is as used before. Preferred genes according to this aspect of the invention include, without limitation, AAD34561, abaA, ACE2, ADR1, AFL1, aflR, AFT1, amyR, areA, ASH1, BAP2, BCY1, CAT8, CDC24, CDC25, CDC28, CDC42, CDC55, CLB2, creA, CTS1, CUP9, CYR1, DFG16, DHH1, DPH3,

ELM1, facB, FLO1, FLO11, FLO8, FUS3, GCN2, GCN4, GCR1, GCR2, GLN3, GPA1, GPA2, GPR1, GRR1, GTS1, HAP1, HAP4, HIP1, HMS1, HMS2, HOG1, HSL1, HXK2, IME1, IME4, INO2, INV11, INV13, INV16, INV5, INV7, INV9, KSS1, LEU3, lovE, LYS14, MAC1, MCM1, MEP1, MEP2, MET28, MET31, MET4, metR, MGA1, MIG1, MIG2, MSN1, MSN2, MSN4, MSN5, MSS11, MTH1, NPR1, nreB, NRG1, OAF1, pacC, PBS2, PDE2, PET9, PHD1, PHO2, PHO4, PHO85, pkaR, PPR1, PTC1, PUT3, RAS1, RAS2, RGS2, RIM101, RIM13, RIM15, RIM9, ROX1, RRE1, SCH9, sconB, SFL1, SHO1, SHR3, SIN3, SIP4, SKN7, SNF1, SNF2, SNF7, SNF8, SOK2, SRB10, SRB11, SRB8, SRB9, sreA, sreP, SRV2, SSD1, SSN6, SST2, STE11, STE12, STE20, STE50, STE7, STP22, SWI4, SWI6, tamA, TEC1, TPK1, TPK2, TPK3, TUP1, UaY, UGA3, URE2, VPS28, VPS36, WHI3, YMR077c, YNL255c, YPR1, ZAP1, genes encoding bacterial protein toxins, and any fungal homologs of the aforementioned genes.

In certain embodiments of the methods according to this aspect of the invention, the modulation is expression of a dominant mutation of the gene. The term "dominant mutation" is as used before. Preferred dominant mutations according to this aspect of the invention are as used before.

In certain embodiments of the methods according to this aspect of the invention, the modulation is mediated by a peptide modulator of gene expression. The term "peptide" is as used before. Peptides may be expressed in the cell or supplied exogenously. Preferably, they are provided on a scaffold to increase intracellular stability and to provide conformational constraint. Preferred peptides according to this aspect of the invention include those discussed earlier.

In certain embodiments of the methods according to this aspect of the invention, the peptide modulator is an activator of gene expression. The term "activator of gene expression" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the peptide modulator is an inhibitor of gene expression. The term "inhibitor of gene expression" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the modulation is mediated by a small molecule modulator of gene expression. In certain embodiments of the methods according to this aspect of the invention, the small molecule modulator is an activator of gene expression. The term "activator of gene expression" is as used

before. In certain embodiments of the methods according to this aspect of the invention, the small molecule modulator is an inhibitor of gene expression. The term "inhibitor of gene expression" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the modulation is conditional expression of the gene. The term "conditional expression" of a gene is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a transcription factor or the product that it encodes acts on a transcription factor. The term "the gene acts on" is as used before. The term "transcription factor" is as used before. Preferred transcription factors include, without limitation, transcription factors that modulate the expression of genes involved in the production or response to the small molecule cAMP (preferred examples include, without limitation, MGA1, MSN2, MSN4, SFL1, and SOK2); transcription factors that function downstream of mitogen-activated protein (MAP) kinase signaling pathways that regulate the yeast invasion response (preferred examples include, without limitation, MCM1, STE12, and TEC1); transcription factors that modulate the expression of genes involved in nitrogen regulation (preferred examples include, without limitation, areA, GLN3, HMS1, HMS2, nreB, tamA, and UGA3); transcription factors that modulate the expression of genes involved in pH regulation in fungi (preferred examples include, without limitation pacC and RIM101); general transcription factors (preferred examples include, without limitation, SIN3, SNF2, SRB8, SRB9, SRB10, SRB11, SSN6, and TUP1); transcription factors that modulate the expression of genes involved in carbon metabolism (preferred examples include, without limitation, ADR1, CAT8, creA, facB, GCR1, GCR2, HAP4, MIG1, MIG2, MTH1, NRG1, OAF1, and SIP4); heme-dependent transcription factors (preferred examples include, without limitation, HAP1 and ROX1); transcription factors that modulate the expression of genes involved in the uptake of metals (preferred examples include, without limitation, AFT1, CUP9, MAC1, sreP, sreA, and ZAP1); transcription factors that modulate the expression of genes involved in cell-cycle regulation (preferred examples include, without limitation, SKN7, SWI4, and SWI6); transcription factors that modulate the expression of genes involved in invasion (preferred examples include, without limitation, ASH1, FLO8, GTS1, INV7, MSN1, MSS11, PHD1, and RRE1); transcription factors that modulate the expression of genes involved

in amino acid biosynthesis or transport (preferred examples include, without limitation, GCN4, LEU3, LYS14, MET4, MET28, MET31, metR, PUT3, sconB, and UGA3); transcription factors that modulate the expression of genes involved in phosphate metabolism or transport (preferred examples include, without limitation, PHO2 and PHO4); transcription factors that modulate the expression of genes involved in nucleotide metabolism or transport (preferred examples include, without limitation, PPR1 and UaY); transcription factors that modulate the expression of genes involved in cell wall processes (preferred examples include, without limitation, ACE2, SWI4, and SWI6); transcription factors that modulate the expression of genes involved in sporulation (preferred examples include, without limitation, IME1 and IME4); transcription factors that modulate the expression of genes involved in phospholipid synthesis (preferred examples include, without limitation, INO2); transcription factors that modulate the expression of genes involved in aflatoxin biosynthesis (preferred examples include, without limitation, aflR); transcription factors that modulate the expression of genes involved in lovastatin biosynthesis (preferred examples include, without limitation, AAD34561 and lovE); and transcription factors that modulate the expression of genes involved in filamentous fungal development (preferred examples include, without limitation, abaA). The term "general transcription factors" is as used before. The term "invasion" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a transmembrane transporter or the product that it encodes acts on a transmembrane transporter. The term "transmembrane transporter" is as used before. Preferred classes of transmembrane transporters include, without limitation, proteins of the ATP-binding cassette superfamily, members of the Major Facilitator Superfamily (MFS), P-type ATPases, members of the mitochondrial carrier family (MCF) that include, without limitation, PET9; ion channels, permeases that include, without limitation, BAP2, HIP1, MEP1, and MEP2; and components that transport sugars, ions, or metals.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a kinase or the product that it encodes acts on a kinase. The term "kinase" is as used before. Preferred kinases include, without limitation, CDC28, ELM1, FUS3, GCN2, HOG1, HSL1, HXK2, KSS1, PBS2, PHO85, RIM15, STE7, SCH9, SNF1, STE11, STE20, TPK1, TPK2, and TPK3.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a G-protein or the product that it encodes acts on a G-protein. The term "G-protein" is as used before. Preferred G-proteins include, without limitation CDC42, fadA, GPA1, GPA2, RAS1, and RAS2.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a cell surface receptor or the product that it encodes acts on a cell surface receptor. The term "cell surface receptor" is as used before. Preferred cell surface receptors include, without limitation, G-protein coupled receptors. Preferred G-protein coupled receptors include, without limitation, GPR1.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a GTPase activating protein or the product that it encodes acts on a GTPase activating protein. The term "GTPase activating protein" is as used before. Preferred GTPase activating proteins include, without limitation, RGS family members. "RGS family members" are regulators of G-protein signaling that act upon G-protein coupled receptors. Preferred RGS family members include, without limitation, FlbA, RGS2, and SST2.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a guanine nucleotide exchange factor or the product that it encodes acts on a guanine nucleotide exchange factor. The term "guanine nucleotide exchange factor" is as used before. Preferred guanine nucleotide exchange factors include, without limitation, CDC24 and CDC25.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a phosphatase or the product that it encodes acts on a phosphatase. The term "phosphatase" is as used before. Preferred phosphatases include, without limitation, CDC55 and PTC1.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a protease or the product that it encodes acts on a protease. The term "protease" is as used before. Preferred proteases include, without limitation, RIM13.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a cyclic nucleotide phosphodiesterase or the product that it encodes acts on a cyclic nucleotide phosphodiesterase. The term "cyclic nucleotide phosphodiesterase" is as used

before. Preferred examples of cyclic nucleotide phosphodiesterases include, without limitation, PDE2.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a bacterial protein toxin or the product that it encodes acts on a bacterial protein toxin. The term "bacterial protein toxin" is as used before. Preferred bacterial protein toxins include, without limitation, Anthrax toxin edema factor (EF; *Bacillus anthracis*), Anthrax toxin lethal factor (LF; *Bacillus anthracis*), adenylate cyclase toxin (*Bordetella pertussis*), Cholera enterotoxin (*Vibrio cholerae*), LT toxin (*Escherichia coli*), ST toxin (*E. coli*), Shiga toxin (*Shigella dysenteriae*), Perfringens enterotoxin (*Clostridium perfringens*), Botulinum toxin (*Clostridium botulinum*), Tetanus toxin (*Clostridium tetani*), Diphtheria toxin (*Corynebacterium diphtheriae*), Exotoxin A (*Pseudomonas aeruginosa*), Exoenzyme S (*P. aeruginosa*), Pertussis toxin (*Bordetella pertussis*), alpha and epsilon toxins (*C. perfringens*), lethal toxin (LT; *Clostridium sordellii*), toxins A and B (*Clostridium dificile*), and phospholipase C (*Clostridium bifermentans*).

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes an importin protein or the product that it encodes acts on an importin protein. The term "importin" protein is as used before. Preferred examples of importin proteins include, without limitation, MSN5.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a RNA-binding protein or the product that it encodes acts on a RNA-binding protein. Preferred examples of RNA-binding proteins include, without limitation, DHH1 and WHI3.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a component of a SCF complex or the product that it encodes acts on a component of a SCF complex. The term "component of a SCF complex" is as used before. Preferred examples of components of a SCF complex include, without limitation, GRR1.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a biosynthetic enzyme or the product that it encodes acts on a biosynthetic enzyme. The term "biosynthetic enzyme" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-bacterial. The term "anti-bacterial" is as used before. Preferred anti-bacterials include, without limitation, B-lactams. Preferred B-lactams include, without limitation, penicillins and cephalosporins. Preferred penicillins and biosynthetic intermediates include, without limitation, isopenicillin N, 6-aminopenicillanic acid (6-APA), penicillin G, penicillin N, and penicillin V. Preferred cephalosporins and biosynthetic intermediates include, without limitation, deacetoxycephalosporin V (DAOC V), deacetoxycephalosporin C (DAOC), deacetylcephalosporin C (DAC), 7-aminodeacetoxycephalosporanic acid (7-ADCA), cephalosporin C, 7- B -(5-carboxy-5-oxopentanamido)-cephalosporanic acid (keto-AD-7ACA), 7- B -(4-carboxybutanamido)-cephalosporanic acid (GL-7ACA), and 7-aminocephalosporanic acid (7ACA).

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-hypercholesterolemic. An "anti-hypercholesterolemic" is as used before. Preferred anti-hypercholesterolemics include, without limitation, lovastatin, mevastatin, simvastatin, and pravastatin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an immunosuppressant. An "immunosuppressant" is as used before. Preferred immunosuppressants include, without limitation, members of the cyclosporin family and beauverolide L. Preferred cyclosporins include, without limitation, cyclosporin A and cyclosporin C.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an ergot alkaloid. The term "ergot alkaloid" is as used before. Preferred classes of ergot alkaloids include clavine alkaloids, lysergic acids, lysergic acid amides, and ergot peptide alkaloids. Preferred ergot alkaloids include, without limitation, ergotamine, ergosine, ergocristine, ergocryptine, ergocornine, ergotaminine, ergosinine, ergocristinine, ergocryptinine, ergocorninine, ergonovine, ergometrinine, and ergoclavine.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an inhibitor of angiogenesis. The term "inhibitor of angiogenesis" is as used before. Preferred inhibitors of angiogenesis include, without limitation, fumagillin and ovalicin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a glucan synthase inhibitor. The term "glucan synthase inhibitor" is as used before. Preferred glucan synthase inhibitors include, without limitation, echinocandin B, pneumocandin B, aculeacin A, and papulacandin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a member of the gliotoxin family of compounds. The term "gliotoxin family of compounds" is as used before. Preferred members of the "gliotoxin family of compounds" include, without limitation, gliotoxin and aspirochlorine.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a fungal toxin. The term "fungal toxin" is as used before. Preferred fungal toxins include, without limitation, aflatoxins, patulin, zearalenone, cytochalasin, griseofulvin, ergochrome, cercosporin, marticin, xanthocillin, coumarins, tricothecenes, fusidanes, sesterpenes, amatoxins, malformin A, phallotoxins, pentoxin, HC toxin, psilocybin, bufotenine, lysergic acid, sporodesmin, pulcheriminic acid, sordarins, fumonisins, ochratoxin A, and fusaric acid.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a modulator of cell surface receptor signaling. The term "cell surface receptor" is as used before. Preferred modulators of cell surface signaling include, without limitation, the insulin receptor agonist L-783,281 and the cholecystokinin receptor antagonist asperlicin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a plant growth regulator. The term "plant growth regulator" is as used before. Preferred plant growth regulators include, without limitation, cytokinin, auxin, gibberellin, abscisic acid, and ethylene.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a pigment. The term "pigment" is as defined before. Preferred pigments include, without limitation, melanins and carotenoids.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an insecticide. The term "insecticide" is as used before. Preferred insecticides include, without limitation, nodulisporic acid.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-neoplastic compound. The term "anti-neoplastic" compound is as used before. Preferred anti-neoplastic compounds include, without limitation, taxol (paclitaxel) and related taxoids.

In certain embodiments of the methods according to this aspect of the invention, the gene is selected from the group consisting of AAD34561, abaA, ACE2, ADR1, AFL1, aflR, AFT1, amyR, areA, ASH1, BAP2, BCY1, CAT8, CDC24, CDC25, CDC28, CDC42, CDC55, CLB2, creA, CTS1, CUP9, CYR1, DFG16, DHH1, DPH3, ELM1, facB, FLO1, FLO11, FLO8, FUS3, GCN2, GCN4, GCR1, GCR2, GLN3, GPA1, GPA2, GPR1, GRR1, GTS1, HAP1, HAP4, HIP1, HMS1, HMS2, HOG1, HSL1, HXK2, IME1, IME4, INO2, INV11, INV13, INV16, INV5, INV7, INV9, KSS1, LEU3, lovE, LYS14, MAC1, MCM1, MEP1, MEP2, MET28, MET31, MET4, metR, MGA1, MIG1, MIG2, MSN1, MSN2, MSN4, MSN5, MSS11, MTH1, NPR1, nreB, NRG1, OAF1, pacC, PBS2, PDE2, PET9, PHD1, PHO2, PHO4, PHO85, pkaR, PPR1, PTC1, PUT3, RAS1, RAS2, RGS2, RIM101, RIM13, RIM15, RIM9, ROX1, RRE1, SCH9, sconB, SFL1, SHO1, SHR3, SIN3, SIP4, SKN7, SNF1, SNF2, SNF7, SNF8, SOK2, SRB10, SRB11, SRB8, SRB9, sreA, sreP, SRV2, SSD1, SSN6, SST2, STE11, STE12, STE20, STE50, STE7, STP22, SWI4, SWI6, tamA, TEC1, TPK1, TPK2, TPK3, TUP1, UaY, UGA3, URE2, VPS28, VPS36, WHI3, YMR077c, YNL255c, YPR1, ZAP1, genes encoding bacterial protein toxins, and any fungal homologs of the aforementioned genes. The term "fungal homolog" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the methods further comprise purifying the secondary metabolite from a culture of the fungus. The term "purifying" is as used before.

In a fifth aspect, the invention provides methods for improving production of a secondary metabolite in a fungus by altering the characteristics of the fungus in a manner that is beneficial to the production of the secondary metabolite, the method comprising modulating the expression of a gene involved in regulation of secondary metabolite production in a manner that alters the characteristics of the fungus. "Altering the characteristics" means changing the morphology or growth traits of the fungus. Preferred alterations include, without limitation, those alterations

that result in transition of the fungus from the hyphal to yeast form, those alterations that result in transition of the fungus from the yeast to hyphal form, alterations that lead to more or less hyphal branching, alterations that increase or decrease flocculence, adherence, cell buoyancy, surface area of the fungus, cell wall integrity and/or stability, pellet size, ability to grow at higher or lower temperatures, and alterations that increase the saturating growth density of a culture or rate of pellet formation.

In certain embodiments of the methods according to this aspect of the invention, the modulation is overexpression of the gene. "Overexpression of the gene" is as used before. Preferred genes according to this aspect of the invention include, without limitation, AAD34561, abaA, ACE2, ADR1, AFL1, aflR, AFT1, AGA1, AGA2, amyR, areA, ASH1, BAP2, BCY1, BEM1, BEM2, BEM3, BNI1, BUD2, BUD5, CAT8, CDC24, CDC25, CDC28, CDC42, CDC55, CLB2, creA, CTS1, CUP9, CYR1, DFG16, DHH1, DPH3, ELM1, facB, FLO1, FLO10, FLO11, FLO5, FLO8, FLO9, FUS3, GCN2, GCN4, GCR1, GCR2, GIC1, GIC2, GLN3, GPA1, GPA2, GPR1, GRR1, GTS1, HAP1, HAP4, HIP1, HMS1, HMS2, HOG1, HSL1, HXK2, IME1, IME4, INO2, INV11, INV13, INV16, INV5, INV7, INV9, KSS1, LEU3, lovE, LYS14, MAC1, MCM1, MEP1, MEP2, MET28, MET31, MET4, metR, MGA1, MIG1, MIG2, MSN1, MSN2, MSN4, MSN5, MSS11, MTH1, NPR1, nreB, NRG1, OAF1, pacC, PBS2, PDE2, PET9, PHD1, PHO2, PHO4, PHO85, pkaR, PPR1, PTC1, PUT3, RAS1, RAS2, RGA1, RGS2, RHO1, RHO2, RHO3, RHO4, RIM101, RIM13, RIM15, RIM9, ROX1, RRE1, RSR1, SCH9, sconB, SFL1, SHO1, SHR3, SIN3, SIP4, SKN7, SNF1, SNF2, SNF7, SNF8, SOK2, SRB10, SRB11, SRB8, SRB9, sreA, sreP, SRV2, SSD1, SSN6, SST2, STE11, STE12, STE20, STE50, STE7, STP22, SWI4, SWI6, tamA, TEC1, TPK1, TPK2, TPK3, TUP1, UaY, UGA3, URE2, VPS28, VPS36, WHI3, YMR077c, YNL255c, YPR1, ZAP1, genes encoding bacterial protein toxins, and any fungal homologs of the aforementioned genes.

In certain embodiments of the methods according to this aspect of the invention, the modulation is expression of a dominant mutation of the gene. The term "dominant mutation" is as used before. Preferred dominant mutations according to this aspect of the invention are as used before.

In certain embodiments of the methods according to this aspect of the invention, the modulation is mediated by a peptide modulator of gene expression. The term "peptide" is as

used before. Peptides may be expressed in the cell or supplied exogenously. Preferably, they are provided on a scaffold to increase intracellular stability and to provide conformational constraint. Preferred peptides according to this aspect of the invention include those discussed earlier.

In certain embodiments of the methods according to this aspect of the invention, the peptide modulator is an activator of gene expression. The term "activator of gene expression" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the peptide modulator is an inhibitor of gene expression. The term "inhibitor of gene expression" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the modulation is mediated by a small molecule modulator of gene expression. In certain embodiments of the methods according to this aspect of the invention, the small molecule modulator is an activator of gene expression. The term "activator of gene expression" is as used before. In certain embodiments of the methods according to this aspect of the invention, the small molecule modulator is an inhibitor of gene expression. The term "inhibitor of gene expression" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the modulation is conditional expression of the gene. The term "conditional expression" of a gene is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a transcription factor or the product that it encodes acts on a transcription factor. The term "the gene acts on" is as used before. The term "transcription factor" is as used before. Preferred transcription factors include, without limitation, transcription factors that modulate the expression of genes involved in the production or response to the small molecule cAMP (preferred examples include, without limitation, MGA1, MSN2, MSN4, SFL1, and SOK2); transcription factors that function downstream of mitogen-activated protein (MAP) kinase signaling pathways that regulate the yeast invasion response (preferred examples include, without limitation, MCM1, STE12, and TEC1); transcription factors that modulate the expression of genes involved in nitrogen regulation (preferred examples include, without limitation, areA, GLN3, HMS1, HMS2, nreB, tamA, and UGA3); transcription factors that modulate the

expression of genes involved in pH regulation in fungi (preferred examples include, without limitation pacC and RIM101); general transcription factors (preferred examples include, without limitation, SIN3, SNF2, SRB8, SRB9, SRB10, SRB11, SSN6, and TUP1); transcription factors that modulate the expression of genes involved in carbon metabolism (preferred examples include, without limitation, ADR1, CAT8, creA, facB, GCR1, GCR2, HAP4, MIG1, MIG2, MTH1, NRG1, OAF1, and SIP4); heme-dependent transcription factors (preferred examples include, without limitation, HAP1 and ROX1); transcription factors that modulate the expression of genes involved in the uptake of metals (preferred examples include, without limitation, AFT1, CUP9, MAC1, sreP, sreA, and ZAP1); transcription factors that modulate the expression of genes involved in cell-cycle regulation (preferred examples include, without limitation, SKN7, SWI4, and SWI6); transcription factors that modulate the expression of genes involved in invasion (preferred examples include, without limitation, ASH1, FLO8, GTS1, INV7, MSN1, MSS11, PHD1, and RRE1); transcription factors that modulate the expression of genes involved in amino acid biosynthesis or transport (preferred examples include, without limitation, GCN4, LEU3, LYS14, MET4, MET28, MET31, metR, PUT3, sconB, and UGA3); transcription factors that modulate the expression of genes involved in phosphate metabolism or transport (preferred examples include, without limitation, PHO2 and PHO4); transcription factors that modulate the expression of genes involved in nucleotide metabolism or transport (preferred examples include, without limitation, PPR1 and UaY); transcription factors that modulate the expression of genes involved in cell wall processes (preferred examples include, without limitation, ACE2, SWI4, and SWI6); transcription factors that modulate the expression of genes involved in sporulation (preferred examples include, without limitation, IME1 and IME4); transcription factors that modulate the expression of genes involved in phospholipid synthesis (preferred examples include, without limitation, INO2); transcription factors that modulate the expression of genes involved in aflatoxin biosynthesis (preferred examples include, without limitation, aflR); transcription factors that modulate the expression of genes involved in lovastatin biosynthesis (preferred examples include, without limitation, AAD34561 and lovE); and transcription factors that modulate the expression of genes involved in filamentous fungal development (preferred examples include, without limitation, abaA). The term "general transcription factors" is as used before. The term "invasion" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a transmembrane transporter or the product that it encodes acts on a transmembrane transporter. The term "transmembrane transporter" is as used before. Preferred classes of transmembrane transporters include, without limitation, proteins of the ATP-binding cassette superfamily, members of the Major Facilitator Superfamily (MFS), P-type ATPases, members of the mitochondrial carrier family (MCF) that include, without limitation, PET9; ion channels, permeases that include, without limitation, BAP2, HIP1, MEP1, and MEP2; and components that transport sugars, ions, or metals.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a kinase or the product that it encodes acts on a kinase. The term "kinase" is as used before. Preferred kinases include, without limitation, CDC28, ELM1, FUS3, GCN2, HOG1, HSL1, HXK2, KSS1, PBS2, PHO85, RIM15, STE7, SCH9, SNF1, STE11, STE20, TPK1, TPK2, and TPK3.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a G-protein or the product that it encodes acts on a G-protein. The term "G-protein" is as used before. Preferred G-proteins include, without limitation CDC42, fadA, GPA1, GPA2, RAS1, RAS2, RHO1, RHO2, RHO3, RHO4, and RSR1.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a cell surface receptor or the product that it encodes acts on a cell surface receptor. The term "cell surface receptor" is as used before. Preferred cell surface receptors include, without limitation, G-protein coupled receptors. Preferred G-protein coupled receptors include, without limitation, GPR1.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a GTPase activating protein or the product that it encodes acts on a GTPase activating protein. The term "GTPase activating protein" is as used before. Preferred GTPase activating proteins include, without limitation, RGS family members. The term "RGS family members" is as used before. Preferred RGS family members include, without limitation, FlbA, RGS2, and SST2. Preferred examples of non-RGS family GTPase-activating proteins include, without limitation, BEM2, BEM3, BUD2, RGA1, and RGA2.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a guanine nucleotide exchange factor or the product that it encodes acts on a guanine nucleotide exchange factor. The term "guanine nucleotide exchange factor" is as used before. Preferred guanine nucleotide exchange factors include, without limitation, BUD5, CDC24, and CDC25.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a phosphatase or the product that it encodes acts on a phosphatase. The term "phosphatase" is as used before. Preferred phosphatases include, without limitation, CDC55 and PTC1.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a protease or the product that it encodes acts on a protease. The term "protease" is as used before. Preferred proteases include, without limitation, RIM13.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a cyclic nucleotide phosphodiesterase or the product that it encodes acts on a cyclic nucleotide phosphodiesterase. The term "cyclic nucleotide phosphodiesterase" is as used before. Preferred examples of cyclic nucleotide phosphodiesterases include, without limitation, PDE2.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a bacterial protein toxin or the product that it encodes acts on a bacterial protein toxin. The term "bacterial protein toxin" is as used before. Preferred bacterial protein toxins include, without limitation, Anthrax toxin edema factor (EF; *Bacillus anthracis*), Anthrax toxin lethal factor (LF; *Bacillus anthracis*), adenylate cyclase toxin (*Bordetella pertussis*), Cholera enterotoxin (*Vibrio cholerae*), LT toxin (*Escherichia coli*), ST toxin (*E. coli*), Shiga toxin (*Shigella dysenteriae*), Perfringens enterotoxin (*Clostridium perfringens*), Botulinum toxin (*Clostridium botulinum*), Tetanus toxin (*Clostridium tetani*), Diphtheria toxin (*Corynebacterium diphtheriae*), Exotoxin A (*Pseudomonas aeruginosa*), Exoenzyme S (*P. aeruginosa*), Pertussis toxin (*Bordetella pertussis*), alpha and epsilon toxins (*C. perfringens*), lethal toxin (LT; *Clostridium sordellii*), toxins A and B (*Clostridium dificile*), and phospholipase C (*Clostridium bifermentans*).

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes an importin protein or the product that it encodes acts on an importin protein. The term "importin" protein is as used before. Preferred examples of importin proteins include, without limitation, MSN5.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a RNA-binding protein or the product that it encodes acts on a RNA-binding protein. Preferred examples of RNA-binding proteins include, without limitation, DHH1 and WHI3.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a component of a SCF complex or the product that it encodes acts on a component of a SCF complex. The term "component of a SCF complex" is as used before. Preferred examples of components of a SCF complex include, without limitation, GRR1.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes an adherin or the product that it encodes acts on an adherin. The term "adherin" means a molecule that functions to promote the interaction of a cell with another component, including, without limitation, interaction with other cells of the same genotype, interaction with cells of a different genotype, and interaction with growth substrates. Preferred examples of adherins include, without limitation, AGA1, AGA2, FLO1, FLO10, FLO11, FLO5, and FLO9.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a biosynthetic enzyme or the product that it encodes acts on a biosynthetic enzyme. The term "biosynthetic enzyme" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-bacterial. The term "anti-bacterial" is as used before. Preferred anti-bacterials include, without limitation, B-lactams. Preferred B-lactams include, without limitation, penicillins and cephalosporins. Preferred penicillins and biosynthetic intermediates include, without limitation, isopenicillin N, 6-aminopenicillanic acid (6-APA), penicillin G, penicillin N, and penicillin V. Preferred cephalosporins and biosynthetic intermediates include, without limitation, deacetoxycephalosporin V (DAOC V), deacetoxycephalosporin C (DAOC), deacetylcephalosporin C (DAC), 7-aminodeacetoxycephalosporanic acid (7-ADCA), cephalosporin C, 7- B -(5-carboxy-5-oxopentanamido)-cephalosporanic acid (keto-AD-7ACA),

7-B-(4-carboxybutanamido)-cephalosporanic acid (GL-7ACA), and 7-aminocephalosporanic acid (7ACA).

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-hypercholesterolemic. An "anti-hypercholesterolemic " is as used before. Preferred anti-hypercholesterolemics include, without limitation, lovastatin, mevastatin, simvastatin, and pravastatin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an immunosuppressant. An "immunosuppressant" is as used before. Preferred immunosuppressants include, without limitation, members of the cyclosporin family and beauverolide L. Preferred cyclosporins include, without limitation, cyclosporin A and cyclosporin C.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an ergot alkaloid. The term "ergot alkaloid" is as used before. Preferred classes of ergot alkaloids include clavine alkaloids, lysergic acids, lysergic acid amides, and ergot peptide alkaloids. Preferred ergot alkaloids include, without limitation, ergotamine, ergosine, ergocristine, ergocryptine, ergocornine, ergotaminine, ergosinine, ergocristinine, ergocryptinine, ergocorninine, ergonovine, ergometrinine, and ergoclavine.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an inhibitor of angiogenesis. The term "inhibitor of angiogenesis" is as used before. Preferred inhibitors of angiogenesis include, without limitation, fumagillin and ovalicin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a glucan synthase inhibitor. The term "glucan synthase inhibitor" is as used before. Preferred glucan synthase inhibitors include, without limitation, echinocandin B, pneumocandin B, aculeacin A, and papulacandin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a member of the gliotoxin family of compounds. The term "gliotoxin family of compounds" is as used before. Preferred members of the "gliotoxin family of compounds" include, without limitation, gliotoxin and aspirochlorine.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a fungal toxin. The term "fungal toxin" is as used before. Preferred fungal toxins include, without limitation, aflatoxins, patulin, zearalenone, cytochalasin, griseofulvin, ergochrome, cercosporin, marticin, xanthocillin, coumarins, tricothecenes, fusidanes, sesterpenes, amatoxins, malformin A, phallotoxins, pentoxin, HC toxin, psilocybin, bufotenine, lysergic acid, sporodesmin, pulcheriminic acid, sordarins, fumonisins, ochratoxin A, and fusaric acid.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a modulator of cell surface receptor signaling. The term "cell surface receptor" is as used before. Preferred modulators of cell surface signaling include, without limitation, the insulin receptor agonist L-783,281 and the cholecystokinin receptor antagonist asperlicin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a plant growth regulator. The term "plant growth regulator" is as used before. Preferred plant growth regulators include, without limitation, cytokinin, auxin, gibberellin, abscisic acid, and ethylene.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a pigment. The term "pigment" is as defined before. Preferred pigments include, without limitation, melanins and carotenoids.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an insecticide. The term "insecticide" is as used before. Preferred insecticides include, without limitation, nodulisporic acid.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-neoplastic compound. The term "anti-neoplastic" compound is as used before. Preferred anti-neoplastic compounds include, without limitation, taxol (paclitaxel) and related taxoids.

In certain embodiments of the methods according to this aspect of the invention, the gene is selected from the group consisting of AAD34561, abaA, ACE2, ADR1, AFL1, aflR, AFT1, AGA1, AGA2, amyR, areA, ASH1, BAP2, BCY1, BEM1, BEM2, BEM3, BNI1, BUD2, BUD5,

~~CAT8, CDC24, CDC25, CDC28, CDC42, CDC55, CLB2, creA, CTS1, CUP9, CYR1, DFG16,~~ DHH1, DPH3, ELM1, facB, FLO1, FLO10, FLO11, FLO5, FLO8, FLO9, FUS3, GCN2, GCN4, GCR1, GCR2, GIC1, GIC2, GLN3, GPA1, GPA2, GPR1, GRR1, GTS1, HAP1, HAP4, HIP1, HMS1, HMS2, HOG1, HSL1, HXK2, IME1, IME4, INO2, INV11, INV13, INV16, INV5, INV7, INV9, KSS1, LEU3, lovE, LYS14, MAC1, MCM1, MEP1, MEP2, MET28, MET31, MET4, metR, MGA1, MIG1, MIG2, MSN1, MSN2, MSN4, MSN5, MSS11, MTH1, NPR1, nreB, NRG1, OAF1, pacC, PBS2, PDE2, PET9, PHD1, PHO2, PHO4, PHO85, pkaR, PPR1, PTC1, PUT3, RAS1, RAS2, RGA1, RGS2, RHO1, RHO2, RHO3, RHO4, RIM101, RIM13, RIM15, RIM9, ROX1, RRE1, RSR1, SCH9, sconB, SFL1, SHO1, SHR3, SIN3, SIP4, SKN7, SNF1, SNF2, SNF7, SNF8, SOK2, SRB10, SRB11, SRB8, SRB9, sreA, sreP, SRV2, SSD1, SSN6, SST2, STE11, STE12, STE20, STE50, STE7, STP22, SWI4, SWI6, tamA, TEC1, TPK1, TPK2, TPK3, TUP1, UaY, UGA3, URE2, VPS28, VPS36, WHI3, YMR077c, YNL255c, YPR1, ZAP1, genes encoding bacterial protein toxins, and any fungal homologs of the aforementioned genes. The term "fungal homolog" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the methods further comprise purifying the secondary metabolite from a culture of the fungus. The term "purifying" is as used before.

In a sixth aspect, the invention provides methods for improving production of a secondary metabolite in a fungus by causing conditional lysis of the fungus, the method comprising modulating the expression of a gene involved in regulation of secondary metabolite production in a manner that causes conditional lysis. "Causing conditional lysis" means causing the fungus to grow without lysis under a first set of growth conditions and to lyse under a second and different set of conditions, which are not lytic to the unmodified fungus. In preferred embodiments, the conditions that can be altered between the first and second growth conditions include, without limitation, the source or amount of nutrients such as carbon, nitrogen, and phosphate; the source or amount of specific enzymes; the source or amount of specific components found in cell walls; the amount of salts or osmolytes; the pH of the medium, the partial oxygen pressure, or temperature; and the amount of specific small molecules.

In certain embodiments of the methods according to this aspect of the invention, the modulation is overexpression of the gene. "Overexpression of the gene" is as used before. Preferred genes according to this aspect of the invention include, without limitation, ACE2, BCK1, BGL2, CHS1, CHS2, CHS3, CTS1, FKS1, GSC2, HOG1, ISR1, KRE6, MID2, MKK1, MKK2, PBS2, PKC1, PPH21, PPH22, PPZ1, PPZ2, PTP2, PTP3, RHO1, RLM1, ROM1, ROM2, SHO1, SKN1, SLG1, SLN1, SLT2, SMP1, SSK1, SSK2, SSK22, STE11, STT3, STT4, SWI4, SWI6, VPS45, WSC2, WSC3, WSC4, and YPD1.

In certain embodiments of the methods according to this aspect of the invention, the modulation is expression of a dominant mutation of the gene. The term "dominant mutation" is as used before. Preferred dominant mutations according to this aspect of the invention are as used before.

In certain embodiments of the methods according to this aspect of the invention, the modulation is mediated by a peptide modulator of gene expression. The term "peptide" is as used before. Peptides may be expressed in the cell or supplied exogenously. Preferably, they are provided on a scaffold to increase intracellular stability and to provide conformational constraint. Preferred peptides according to this aspect of the invention include those discussed earlier.

In certain embodiments of the methods according to this aspect of the invention, the peptide modulator is an activator of gene expression. The term "activator of gene expression" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the peptide modulator is an inhibitor of gene expression. The term "inhibitor of gene expression" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the modulation is mediated by a small molecule modulator of gene expression. In certain embodiments of the methods according to this aspect of the invention, the small molecule modulator is an activator of gene expression. The term "activator of gene expression" is as used before. In certain embodiments of the methods according to this aspect of the invention, the small molecule modulator is an inhibitor of gene expression. The term "inhibitor of gene expression" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the modulation is conditional expression of the gene. The term "conditional expression" of a gene is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a transcription factor or the product that it encodes acts on a transcription factor. The term "transcription factor" is as used before. Preferred transcription factors include, without limitation, ACE2, RLM1, SMP1, SWI4, and SWI6.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a transmembrane transporter or the product that it encodes acts on a transmembrane transporter. A "transmembrane transporter" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a kinase or the product that it encodes acts on a kinase. A "kinase" is as used before. Preferred kinases include, without limitation, BCK1, HOG1, ISR1, MKK1, MKK2, PBS2, PKC1, SLT2, SSK2, and SSK22.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a component involved in cell wall biosynthesis or the product that it encodes acts on a component involved in cell wall biosynthesis. Preferred classes of components involved in cell wall biosynthesis include, without limitation, glucan synthases, glucanases, chitin synthase, and chitinases. Preferred examples of components involved in cell wall biosynthesis include, without limitation, BGL2, CHS1, CHS2, CHS3, CTS1, FKS1, GSC2, KRE6, and SKN1.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a G-protein or the product that it encodes acts on a G-protein. A "G-protein" is a guanyl-nucleotide binding protein. Preferred G-proteins include, without limitation RHO1.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a cell surface receptor or the product that it encodes acts on a cell surface receptor. A "cell surface receptor" is as used before. Preferred cell surface receptors include, without limitation, SHO1 and SLN1.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a GTPase activating protein or the product that it encodes acts on a GTPase activating protein. A "GTPase activating protein" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a guanine nucleotide exchange factor or the product that it encodes acts on a guanine nucleotide exchange factor. A "guanine nucleotide exchange factor" is as used before. Preferred guanine nucleotide exchange factors include, without limitation, ROM1 and ROM2.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a phosphatase or the product that it encodes acts on a phosphatase. A "phosphatase" is as used before. Preferred phosphatases include, without limitation, PPH21, PPH22, PPZ1, PPZ2, PTP2, PTP3, and PTC1.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a protease or the product that it encodes acts on a protease. A "protease" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a cyclic nucleotide phosphodiesterase or the product that it encodes acts on a cyclic nucleotide phosphodiesterase. A "cyclic nucleotide phosphodiesterase" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a bacterial protein toxin or the product that it encodes acts on a bacterial protein toxin. A "bacterial protein toxin" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes an importin or the product that it encodes acts on an importin protein. An "importin" protein is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a RNA-binding protein or the product that it encodes acts on a RNA-binding protein.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a component of a SCF complex or the product that it encodes acts on a component of a SCF complex. A "component of a SCF complex" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a biosynthetic or the product that it encodes acts on a biosynthetic enzyme. The term "biosynthetic enzyme" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-bacterial. The term "anti-bacterial" is as used before. Preferred anti-bacterials include, without limitation, B-lactams. Preferred B-lactams include, without limitation, penicillins and cephalosporins. Preferred penicillins and biosynthetic intermediates include, without limitation, isopenicillin N, 6-aminopenicillanic acid (6-APA), penicillin G, penicillin N, and penicillin V. Preferred cephalosporins and biosynthetic intermediates include, without limitation, deacetoxycephalosporin V (DAOC V), deacetoxycephalosporin C (DAOC), deacetylcephalosporin C (DAC), 7-aminodeacetoxycephalosporanic acid (7-ADCA), cephalosporin C, 7- B -(5-carboxy-5-oxopentanamido)-cephalosporanic acid (keto-AD-7ACA), 7- B -(4-carboxybutanamido)-cephalosporanic acid (GL-7ACA), and 7-aminocephalosporanic acid (7ACA).

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-hypercholesterolemic. An "anti-hypercholesterolemic" is as used before. Preferred anti-hypercholesterolemics include, without limitation, lovastatin, mevastatin, simvastatin, and pravastatin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an immunosuppressant. An "immunosuppressant" is as used before. Preferred immunosuppressants include, without limitation, members of the cyclosporin family and beauverolide L. Preferred cyclosporins include, without limitation, cyclosporin A and cyclosporin C.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an ergot alkaloid. The term "ergot alkaloid" is as used before. Preferred classes of ergot alkaloids include clavine alkaloids, lysergic acids, lysergic acid amides, and ergot peptide alkaloids. Preferred ergot alkaloids include, without limitation, ergotamine, ergosine, ergocristine, ergocryptine, ergocornine, ergotaminine, ergosinine, ergocristinine, ergocryptinine, ergocorninine, ergonovine, ergometrinine, and ergoclavine.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an inhibitor of angiogenesis. The term "inhibitor of angiogenesis" is as used before. Preferred inhibitors of angiogenesis include, without limitation, fumagillin and ovalicin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a glucan synthase inhibitor. The term "glucan synthase inhibitor" is as used before. Preferred glucan synthase inhibitors include, without limitation, echinocandin B, pneumocandin B, aculeacin A, and papulacandin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a member of the gliotoxin family of compounds. The term "gliotoxin family of compounds" is as used before. Preferred members of the "gliotoxin family of compounds" include, without limitation, gliotoxin and aspirochlorine.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a fungal toxin. The term "fungal toxin" is as used before. Preferred fungal toxins include, without limitation, aflatoxins, patulin, zearalenone, cytochalasin, griseofulvin, ergochrome, cercosporin, marticin, xanthocillin, coumarins, tricothecenes, fusidanes, sesterpenes, amatoxins, malformin A, phallotoxins, pentoxin, HC toxin, psilocybin, bufotenine, lysergic acid, sporodesmin, pulcheriminic acid, sordarins, fumonisins, ochratoxin A, and fusaric acid.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a modulator of cell surface receptor signaling. The term "cell surface receptor" is as used before. Preferred modulators of cell surface signaling include, without limitation, the insulin receptor agonist L-783,281 and the cholecystokinin receptor antagonist asperlicin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a plant growth regulator. The term "plant growth regulator" is as used before. Preferred plant growth regulators include, without limitation, cytokinin, auxin, gibberellin, abscisic acid, and ethylene.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a pigment. The term "pigment" is as defined before. Preferred pigments include, without limitation, melanins and carotenoids.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an insecticide. The term "insecticide" is as used before. Preferred insecticides include, without limitation, nodulisporic acid.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-neoplastic compound. The term "anti-neoplastic" compound is as used before. Preferred anti-neoplastic compounds include, without limitation, taxol (paclitaxel) and related taxoids.

In certain embodiments of the methods according to this aspect of the invention, the gene is selected from the group consisting of ACE2, BCK1, BGL2, CHS1, CHS2, CHS3, CTS1, FKS1, GSC2, HOG1, ISR1, KRE6, MID2, MKK1, MKK2, PBS2, PKC1, PPH21, PPH22, PPZ1, PPZ2, PTP2, PTP3, RHO1, RLM1, ROM1, ROM2, SHO1, SKN1, SLG1, SLN1, SLT2, SMP1, SSK1, SSK2, SSK22, STE11, STT3, STT4, SWI4, SWI6, VPS45, WSC2, WSC3, WSC4, YPD1, and fungal homologs of the aforementioned genes. The term "fungal homolog" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the methods further comprise purifying the secondary metabolite from a culture of the fungus. The term "purifying" is as used before.

In a seventh aspect, the invention provides methods for improving production of a secondary metabolite in a fungus by increasing the resistance of the fungus to the deleterious effects of exposure to a secondary metabolite made by the same organism, the method comprising modulating the expression of a gene involved in regulation of secondary metabolite production in a manner that increases resistance to the deleterious effects of exposure to a secondary metabolite. "Increasing the resistance of the fungus to the deleterious effects of exposure to a secondary metabolite" means to allow the fungus to survive, grow, or produce secondary metabolite in conditions that otherwise would be toxic or prevent production of secondary metabolite.

In certain embodiments of the methods according to this aspect of the invention, the modulation is overexpression of the gene. "Overexpression of the gene" is as used before. Preferred genes according to this aspect of the invention include, without limitation, AAD34558, AAD34561, AAD34564, ATR1, ERG6, ERG11, FCR1, GCN4, lovE, MDR1, PDR1, PDR3, PDR5, PDR10, PDR13, SNQ2, TRI12, YAP1, fungal homologs of the aforementioned genes, and genes that encode beta-tubulin, calcineurin (including, without limitation, CNA1), chitin

synthase, glucan synthase, HMG CoA reductase, N-terminal aminopeptidases, and RNA polymerase II.

In certain embodiments of the methods according to this aspect of the invention, the modulation is expression of a dominant mutation of the gene. The term "dominant mutation" is as used before. Preferred dominant mutations according to this aspect of the invention are as used before.

In certain embodiments of the methods according to this aspect of the invention, the modulation is mediated by a peptide modulator of gene expression. The term "peptide" is as used before. Peptides may be expressed in the cell or supplied exogenously. Preferably, they are provided on a scaffold to increase intracellular stability and to provide conformational constraint. Preferred peptides according to this aspect of the invention include those discussed earlier.

In certain embodiments of the methods according to this aspect of the invention, the peptide modulator is an activator of gene expression. The term "activator of gene expression" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the peptide modulator is an inhibitor of gene expression. The term "inhibitor of gene expression" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the modulation is mediated by a small molecule modulator of gene expression. In certain embodiments of the methods according to this aspect of the invention, the small molecule modulator is an activator of gene expression. The term "activator of gene expression" is as used before. In certain embodiments of the methods according to this aspect of the invention, the small molecule modulator is an inhibitor of gene expression. The term "inhibitor of gene expression" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the modulation is conditional expression of the gene. The term "conditional expression" of a gene is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a transcription factor or the product that it encodes acts on a transcription factor.

The term "transcription factor" is as used before. Preferred transcription factors include, without limitation, AAD34561, FCR1, GCN4, lovE, PDR1, PDR3, and YAP1.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a transmembrane transporter or the product that it encodes acts on a transmembrane transporter. The term "transmembrane transporter" is as used before. Preferred transmembrane transporters include, without limitation, AAD34558, AAD34564, ATR1, MDR1, PDR5, PDR10, SNQ2, and TRI12.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a kinase or the product that it encodes acts on a kinase. A "kinase" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a G-protein or the product that it encodes acts on a G-protein. A "G-protein" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a cell surface receptor or the product that it encodes acts on a cell surface receptor. A "cell surface receptor" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a GTPase activating protein or the product that it encodes acts on a GTPase activating protein. A "GTPase activating protein" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a guanine nucleotide exchange factor or the product that it encodes acts on a guanine nucleotide exchange factor. A "guanine nucleotide exchange factor" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a phosphatase or the product that it encodes acts on a phosphatase. A "phosphatase" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a protease or the product that it encodes acts on a protease. A "protease" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a cyclic nucleotide phosphodiesterase or the product that it encodes acts on a cyclic nucleotide phosphodiesterase. A "cyclic nucleotide phosphodiesterase" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a bacterial protein toxin or the product that it encodes acts on a bacterial protein toxin. A "bacterial protein toxin" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes an importin protein or the product that it encodes acts on an importin protein. An "importin" protein is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a RNA-binding protein or the product that it encodes acts on a RNA-binding protein.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a component of a SCF complex or the product that it encodes acts on a component of a SCF complex. A "component of a SCF complex" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a biosynthetic enzyme or the product that it encodes acts on a biosynthetic enzyme. The term "biosynthetic enzyme" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-bacterial. The term "anti-bacterial" is as used before. Preferred anti-bacterials include, without limitation, B-lactams. Preferred B-lactams include, without limitation, penicillins and cephalosporins. Preferred penicillins and biosynthetic intermediates include, without limitation, isopenicillin N, 6-aminopenicillanic acid (6-APA), penicillin G, penicillin N, and penicillin V. Preferred cephalosporins and biosynthetic intermediates include, without limitation, deacetoxycephalosporin V (DAOC V), deacetoxycephalosporin C (DAOC), deacetylcephalosporin C (DAC), 7-aminodeacetoxycephalosporanic acid (7-ADCA), cephalosporin C, 7- B -(5-carboxy-5-oxopentanamido)-cephalosporanic acid (keto-AD-7ACA), 7- B -(4-carboxybutanamido)-cephalosporanic acid (GL-7ACA), and 7-aminocephalosporanic acid (7ACA).

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-hypercholesterolemic. An "anti-hypercholesterolemic" is as used before. Preferred anti-hypercholesterolemics include, without limitation, lovastatin, mevastatin, simvastatin, and pravastatin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an immunosuppressant. An "immunosuppressant" is as used before. Preferred immunosuppressants include, without limitation, members of the cyclosporin family and beauverolide L. Preferred cyclosporins include, without limitation, cyclosporin A and cyclosporin C.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an ergot alkaloid. The term "ergot alkaloid" is as used before. Preferred classes of ergot alkaloids include clavine alkaloids, lysergic acids, lysergic acid amides, and ergot peptide alkaloids. Preferred ergot alkaloids include, without limitation, ergotamine, ergosine, ergocristine, ergocryptine, ergocornine, ergotaminine, ergosinine, ergocristinine, ergocryptinine, ergocorninine, ergonovine, ergometrinine, and ergoclavine.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an inhibitor of angiogenesis. The term "inhibitor of angiogenesis" is as used before. Preferred inhibitors of angiogenesis include, without limitation, fumagillin and ovalicin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a glucan synthase inhibitor. The term "glucan synthase inhibitor" is as used before. Preferred glucan synthase inhibitors include, without limitation, echinocandin B, pneumocandin B, aculeacin A, and papulacandin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a member of the gliotoxin family of compounds. The term "gliotoxin family of compounds" is as used before. Preferred members of the "gliotoxin family of compounds" include, without limitation, gliotoxin and aspirochlorine.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a fungal toxin. The term "fungal toxin" is as used before. Preferred fungal toxins include, without limitation, aflatoxins, patulin, zearalenone, cytochalasin,

griseofulvin, ergochrome, cercosporin, marticin, xanthocillin, coumarins, tricothecenes, fusidanes, sesterpenes, amatoxins, malformin A, phallotoxins, pentoxin, HC toxin, psilocybin, bufotenine, lysergic acid, sporodesmin, pulcheriminic acid, sordarins, fumonisins, ochratoxin A, and fusaric acid.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a modulator of cell surface receptor signaling. The term "cell surface receptor" is as used before. Preferred modulators of cell surface signaling include, without limitation, the insulin receptor agonist L-783,281 and the cholecystokinin receptor antagonist asperlicin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a plant growth regulator. The term "plant growth regulator" is as used before. Preferred plant growth regulators include, without limitation, cytokinin, auxin, gibberellin, abscisic acid, and ethylene.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a pigment. The term "pigment" is as defined before. Preferred pigments include, without limitation, melanins and carotenoids.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an insecticide. The term "insecticide" is as used before. Preferred insecticides include, without limitation, nodulisporic acid.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-neoplastic compound. The term "anti-neoplastic" compound is as used before. Preferred anti-neoplastic compounds include, without limitation, taxol (paclitaxel) and related taxoids.

In certain embodiments of the methods according to this aspect of the invention, the gene is selected from the group consisting of AAD34558, AAD34561, AAD34564, ATR1, ERG6, ERG11, FCR1, GCN4, lovE, MDR1, PDR1, PDR3, PDR5, PDR10, PDR13, SNQ2, TRI12, YAP1, fungal homologs of the aforementioned genes, and genes that encode beta-tubulin, calcineurin (including, without limitation, CNA1), chitin synthase, glucan synthase, HMG CoA reductase, N-terminal aminopeptidases, and RNA polymerase II.

In certain embodiments of the methods according to this aspect of the invention, the methods further comprise purifying the secondary metabolite from a culture of the fungus. The term "purifying" is as used before.

In an eighth aspect, the invention provides genetically modified fungi, wherein the genetically modified fungi have an ability to produce secondary metabolites and the ability of the genetically modified fungus to produce secondary metabolites has been improved by any of the methods according to the invention.

In a ninth aspect, the invention provides a method for making a secondary metabolite, the method comprising culturing a genetically modified fungus according to the invention under conditions suitable for the production of secondary metabolites. "Conditions suitable for the production of secondary metabolites" means culture conditions under which the fungus does in fact produce one or more secondary metabolite.

The following examples are intended to further illustrate certain preferred embodiments of the invention and are not intended to limit the scope of the invention in any way.

Example 1

Preparation of clones to regulate secondary metabolite production

To prepare clones that can be used to genetically modulate the expression of genes involved in secondary metabolism, the following experiments were conducted.

The Gateway (Life Technologies, Inc.) Cloning Technology (US Patent 5,888,732) was used to generate constructs for expression of fungal regulators. The polymerase chain reaction (PCR) was used to amplify cDNA or genomic DNA containing coding sequence for fungal regulators; the resultant PCR products contain common sites at both 5' and 3' ends in order to facilitate recombination into the Gateway entry vector MB971 (see Life Technologies Inc., www.lifetech.com). The resultant entry clones were then reacted in a Gateway destination cocktail with plasmid MB1419 (or related destination vectors). MB1419 is derived from

pLXZ161. pLXZ161 is a gene vector derived from pBC-phleo (P. Silar, Fungal Genetics Newsletter 42: 73 (1995)) that carries a phleomycin resistance cassette for selection of transformants, as well as a polylinker located between the *Aspergillus nidulans PGK* promoter and the *A. nidulans trpC* terminator. pLXZ161 was constructed as follows: First, the *Aspergillus nidulans trpC* terminator was amplified from *A. nidulans* genomic DNA by PCR using Turbo Pfu Polymerase as described by the manufacturer (Stratagene,11011 North Torrey Pines Road, La Jolla, CA 92037). Primers used in this reaction are TRPC-1 5'-GCGGCCGCGGCGCCCGGCCCATGTCAACAAGAAT-3') and TRPC-2 5'-CCGCGGCCGAGTGGAGATGTGGAGT-3'. The resultant product was digested with the restriction enzymes *Sac*II and *Not*I, purified by agarose gel electrophoresis, and cloned into *Sac*II/*Not*I-digested pBC-phleo DNA, to generate pLXZ116. Second, the *A. nidulans* PGK promoter was amplified from *A. nidulans* genomic DNA by PCR using primers PGK1-1 5'-CATGGGGCCCCGTGATGTCTACCTGCCCAC-3' and PGK1-2 5'-CATGATCGATTGTGGGTAGTTAATGGTATG-3', Turbo Pfu Polymerase, and reaction conditions as described above. The resultant product was digested with *Apa*I and *Cla*I and cloned into *Apa*I/*Cla*I-digested pLXZ116, to generate pLXZ161. To produce MB1419, the *ccdB* (death gene) cassette from pEZC7201 (Life Technologies, Gateway cloning manual) was amplified by PCR using oligos MO511 (GGCCATCGATACAAGTTTGTACAAAAAAGCTGAAC) and MO512 (GCGGCCGCACCACTTTGTACAAGAAAGC), digested with *Cla*I and *Not*I, and cloned into *Not*I/*Cla*I-digested pLXZ161. This generated a destination vector in which the death gene cassette resides between the *A. nidulans PGK* promoter and the *A. nidulans trpC* terminator of pLXZ161. Thus, destination reactions using this vector allow configuration of any gene in an entry clone to be expressed under the control of the *A. nidulans PGK* promoter. The fungal selectable marker contained on this plasmid is *ble*, which confers resistance to phleomycin.

Example 2

Transformation of *Aspergillus terreus* and *Penicillium chrysogenum*

Destination clones were transformed into either *Aspergillus terreus* or *Penicillium chrysogenum*. In order to transform these fungi, spores were first generated by culture of strain

ATCC#20542 (*A. terreus*), MF1 (NRRL1951, *P. chrysogenum*), or MF20 (ATCC#11702, *P. chrysogenum*) on petri plates containing potato dextrose agar (Difco BRL) at 30°C for 3-6 days. Spores were removed from PDA either by resuspension in sterile water or Tween-80 (0.1%) or by scraping directly from the plate using a sterile spatula. Yeast extract sucrose medium, or YES (2% Yeast Extract, 6% Sucrose), was inoculated to a density of 1-5 x 10^6 spores per ml and incubated with shaking in an Erlenmeyer flask at 26-30° C for 12-16 hr (250 rpm). Mycelia were harvested by centrifugation at 3200 rpm for 10 minutes, and washed in sterile water two times. Mycelia were resuspended in a filter sterilized solution of Novozyme 234 (Sigma) at 2-5 mg/ml in 1 M $MgSO_4$ and digested at room temperature with shaking (80 rpm) for 1-2 hr. Undigested material was removed by filtration through Miracloth (Calbiochem, 10394 Pacific Center Court, San Diego, CA 92121). After adding 1-2 volumes of STC (0.8 M sorbitol, 25 mm Tris, pH 7.5, and 25 mM $CaCl_2$), the protoplasts were pelleted by centrifugation at 2500 rpm. Protoplasts were washed 2 times in STC by centrifugation. Resulting protoplasts were resuspended to a density of 5 x 10^7 per ml in a solution of STC, SPTC (40 % polyethylene glycol in STC) and DMSO in a ratio of 9:1:0.1 and frozen at –80°C. For transformations, two aliquots (100 µl each) of protoplasts were mixed with 1-5 ug of either pBCphleo or destination clones for expression of fungal regulators; mixtures were incubated on ice for 30 min. An aliquot of SPTC (15 µl) was added to each tube and the reaction was incubated at room temperature for 15 minutes. An additional aliquot (500 µl) was added with gentle mixing, and the reaction was incubated for an additional 15 minutes at room temperature. The reaction was next resuspended in 25 ml of molten regeneration medium (Potato Dextrose Agar from Sigma, 3050 Spruce Street, St Louis, MO 63103) with 0.8 M sucrose, maintained at 50° C, and poured onto a 150 mm petri plate containing 25 ml of solidified regeneration medium plus phleomycin (60-200 µg/ml for *A. terreus* and 30 µg/ml for *P. chrysogenum*). Transformants are typically visible after 2-5 days of incubation at 26–30°C.

Phleomycin resistant colonies were colony purified into small 24 well plates and then examined both on plates and in shake flask cultures. Morphological and developmental effects of the transgene were observed under both growth conditions. Due to the heterogeneous nature of transformation in filamentous fungi, at least 10 (and often many more) phleomycin resistant

colonies were pursued. Detailed examination of a subset of phleomycin resistant colonies suggests that approximately 80% of the colonies contain a transgene.

Example 3

Determination of lovastatin production

Lovastatin assays were performed using broths from shake flask cultures of *A. terreus* . *A. terreus* transformants were grown on modified RPM medium (WO/37629) containing 4% glucose, 0.3% corn steep liquor (Sigma), 0.2%KNO_3, 0.3%KH_2PO_4, 0.05%$MgSO_4$.7 H_2O, 0.05% NaCl, 0.05% polyglycol (Dow), 0.1 % trace elements (14.3 g/l $ZnSO_4$.7 H_2O, 2.5 g/l $CuSO_4$.5 H_2O, 0.5 g/l $NiCl_2$.6 H_2O, 13.8 g/l $FeSO_4$.7 H_2O, 8.5 g/l MnSO4. H_2O, 3 g/l citric acid. H_2O (add first), 1 g/l H_3BO_3, 1 g/l Na_2MoO_4, 2.5 g/l $CoCl_2$.6 H_2O). The final pH was adjusted to 6.5. Spores for inoculation were generated by culturing on plates containing minimal medium plus phleomycin for 1 week at 27° C. Spores for shake flask inoculation were removed from plates by dragging the tip of a sterile wooden stick approximately 1 inch across the plate surface. The tip of the stick was then dipped into the shake flask medium and swirled gently. Cultures were grown at 27° C, 225 RPM for 5-6 days.

Quantitative assays were performed to assess the levels of lovastatin in broths from shake flask cultures. To assay lovastatin production, $(His)_6$HMGCoA reductase was first expressed in *Saccharomyces cerevisiae* and purified with a nickel column. *A. terreus* samples were fermented as described above and 0.5 mL samples were taken at day 5-6, put in a 1 mL 96-well plate, and centrifuged to remove mycelia before assaying. Samples were transferred to another 1 mL 96-well plate and frozen at –80°C.

Samples were thawed and 10 µL removed and diluted 1:50 in H_2O. 10 µL of this diluted broth was assayed in a reaction (200 µL total) containing 1 mM L-HMGCoA, 1 mM NADPH, 0.005 mM DTT and 5 µL $(His)_6$HMGCoA reductase. The disappearance of absorbance at 340 nm was observed over time, and this represents the utilization of NADPH, an electron donor required for the reduction of HMGCoA. Lovastatin inhibits HMGCoA reductase, and thus assays containing lovastatin display a decreased rate of disappearance of absorbance at 340 nm. The initial velocities for NADPH disappearance were calculated for broth-containing samples

and reactions containing lovastatin standards. Velocities were then adjusted for dilution, and regression analysis was used to determine metabolite concentration.

Several fungal regulators were found to improve the overall yield of lovastatin in shake flask cultures. It is possible that these regulators will also increase productivity. Lovastatin production levels from strains containing regulators were compared to either levels from strains containing control vector or a non-transformed strain. Data points were collected for at least 10 phleomycin resistant colonies, and the production levels for each sample set was displayed as a box plot (*e.g.*, Figure 3). In box plot portrayals of the data, the box represents the central 50% of the data, and the line within the box represents the median value for the entire data set; outlying data points are flagged. Box plot portrayals assist in determining whether a particular sample set is significantly different from a set collected from a control strain.

Table 1 displays representative fungal regulators that improved the yield of lovastatin in shake flask cultures.

Table 1.

Plasmid Name	Regulator
MB1423	pacC (DNA-binding domain (DBD))-VP16 (transcription activation domain (TAD))
MB1695	VP16 (TAD)-pacC (DBD)
MB1564	VP16 (TAD)-pacCL266
MB2415	amdAG229D (TAD)-pacCL266
MB2417	amdAG229C (TAD)-pacCL266
MB2418	amdAG229D (TAD)-pacC (DBD)
MB2419	amdAG229C (TAD)-pacC (DBD)
MB2203	VP16 (TAD)-An09
MB1316	lovE
MB2244	VP16 (TAD)-Pc23
MB1970	At18
MB1310	creA

Box plots are displayed in Figure 3. Hutchinson *et al.*, PCT Publication WO 00/37629, has demonstrated that overexpression of lovE increases lovastatin production in *Aspergillus terreus*; thus, lovE expressing strains served as positive controls in these experiments. The data in Figure 3 is organized in sets of three; samples expressing a particular regulator are always compared to control samples (both positive and negative) grown and assayed at the same time.

The results in Figure 3 indicate that several fungal regulators appreciably stimulate production of lovastatin.

Example 4

Determination of penicillin production

Penicillin assays were performed using broths from shake flask cultures of *P. chrysogenum*. To test levels of penicillin produced in *P. chrysogenum* transformants, a plug containing spores and mycelia was used as the inoculum. The published P2 production medium (J Lein (1986) in Overproduction of microbial metabolites (Z. Vanek and Z. Hostalek eds.) pp. 105-139), which contains, 30% lactose, 5X pharmamedia cotton seed flour, ammonium sulfate, calcium carbonate, potassium phosphate, potassium sulfate, and phenoxyacetic acid pH 7, was used. Flasks were incubated at 26° C with shaking at 225 rpm, and sampling was done after 6 days of growth.

To monitor penicillin production, 1-1.5 mls of broth was placed into 96-well plates. The fermentation broth was clarified by centrifugation for 10 min at 4000 g. Supernatants were transferred to a new 96-well plate. Standard samples containing 0, 25, 50, 100, 200, 300, 400, 500 μg/mL phenoxymethylpenicillin (sodium salt) were dissolved in 10 mM potassium phosphate (pH 7.0). For penicillin assays 40 μL of clarified fermentation broth and penicillin standard solutions were transferred to a 96-well UV, collection plate. 200 μL of imidazole reagent was placed in a 96-well filter plate (0.45 micron). The imidazole reagent was prepared by dissolving 8.25 g of imidazole in 60 mL of water, adding 10 mL of 5 M HCl and then adding 10 mL of mercuric chloride solution (0.27 g dissolved in 100 mL of water). The pH of the imidazole reagent was adjusted to 6.80 +/- 0.05 with 5 M HCl and then diluted to 100 mL with water (see *e.g.*, Bundgaard, H. and K. Ilver, Journal of Pharm Pharmac 24: 790-794 (1972)). The derivatization reaction of penicillin was initiated by vacuum filtration of imidazole reagent into a collection plate containing the aliquoted samples and standards. The collection plate was placed into the 96-well plate reader at 45°C, and an increase at 325 nm was monitored over 20 minutes. A Molecular Devices 96-well UV/Vis plate reader was used for all spectrophotometric detection.

Several fungal regulators were found to improve the yield of penicillin in shake flask cultures. These experiments were performed in both MF1 (NRRL1951), an early strain in the

penicillin development series, and MF20 (ATCC#11702), a strain of *Penicillium chrysogenum* that produces approximately ten-fold more penicillin than MF1. As described above for lovastatin, large numbers of phleomycin resistant colonies were used in shake flask experiments, such that analysis could be performed to determine whether the effect of a particular regulator was statistically significant. Strains of MF20 expressing pacCL266 (MB1563), an alkalinity mimicking allele of pacC, displayed increased penicillin production. pacC (DBD)-VP16 (TAD) (*e.g.*, MB1423) stimulated penicillin production in MF1. In addition, both shake flask and small-scale bioreactor studies demonstrate that this regulator can improve the productivity of Penicillium strains; strains expressing pacC (DBD)-VP16 (TAD) initiate production and reach maximum production levels earlier than the parent MF1 strain or a strain transformed with a control vector. Regulators from fungi other than *Penicillium chrysogenum* also were found to improve penicillin production. Both MF1 and MF20 strains that expressed *lovU* (MB1317), a gene from *Aspergillus terreus*, displayed increased yields of penicillin production. Penicillin yields were also improved in MF20 strains that expressed *YHR056c*, a gene from *Saccharomyces cerevisiae*.

These results demonstrate that many fungal regulator genes are capable of improving penicillin productions, including genes from unrelated species.

Example 5

Alteration of fungal morphology

In addition to improving yield or productivity, several other traits can be modulated in order to improve the process of production of secondary metabolites in fungi. Desired traits would include altering morphological characteristics that would be favorable to a particular fermentation. Several fungal regulators were found to alter morphological or developmental characteristics of *Penicillium* strains. Specifically, pacC (DBD)-VP16 (TAD) (*e.g.*, MB1423) and VP16 (TAD)-areA (from *Penicillium chrysogenum)* (MB2220) caused hyphae to aggregate in shake flask cultures. Pellet size is often a critical factor during growth in bioreactors. Pellet size can impact variables during growth such as the amount of energy needed to drive the impellers within the bioreactor. Aggregating cultures also can be beneficial for purification of biomass from culture broth during post-fermentation processing. In addition to these

morphological effects, expression of pacC (DBD)-VP16 (TAD) (*e.g.*, MB1423), VP16 (TAD)-areA (MB2220), At32 (from *Aspergillus terreus*) (MB1623), and VP16 (TAD)-At32 (MB2200) affected the developmental process of sporulation. Strains expressing pacC (DBD)-VP16 (TAD), VP16 (TAD)-areA, and VP16 (TAD)-At32 are sporulation defective, whereas strains expressing At32 sporulate in submerged culture. In some instances (*e.g.*, sterigmatocystin biosynthesis in *Aspergillus nidulans*) sporulation and production of secondary metabolites are coordinately regulated. In other examples, such as penicillin production, sporulation defective strains often produced increased levels of metabolite. Therefore, regulators that increase or decrease sporulation may provide a tool to adjust the developmental state of the fungus to the optimal state for production of any particular metabolite.

Example 6

Reducing toxic effects of a secondary metabolite

Other desired traits would include increasing resistance to the deleterious effects of exposure to a secondary metabolite,

Growth of a fungus that produces secondary metabolites can be limited, in part, by the toxic effects of the secondary metabolites themselves. In the absence of resistance mechanisms to protect fungi from toxic effects of these metabolites, decreased yields of the metabolite can be observed. For example, Alexander *et al.* (Mol. Gen. Genet. 261: 977-84 (1999)) have shown that the trichothecene efflux pump of *Fusarium sporotrichiodes*, encoded by the gene *TRI12*, is required both for high level production of, and resistance to the toxic effects of, trichothecenes produced by this fungus. Thus, modifications that increase the resistance of a fungus to a toxic secondary metabolite that it produces can increase the saturation density and extend the metabolically active lifetime of the producing fungus. In a bioreactor, such attributes will have the beneficial effect of increasing yield and productivity of a metabolite. Regulators of secondary metabolite production whose expression can be modulated to increase resistance of a fungus to toxic metabolites that it produces can include, without limitation, transporters that promote efflux of the metabolite from cells, enzymes that alter the chemical structure of the metabolite within

cells to render it non-toxic, target(s) of the metabolite that mediate its toxicity, and gene products that alter cellular processes to counteract the toxic effects of a metabolite. Additional benefits of increasing efflux of secondary metabolites include increasing the amount of metabolite available for purification from the fermentation broth and mitigation of feedback inhibition of secondary metabolism that may be mediated by the metabolite itself. Indeed, feedback inhibition of a biosynthetic pathway by a product of that pathway is well documented in many microorganisms, and this inhibition can act at the transcriptional, translational, and post-translational levels. Several well-documented examples in yeast include the transcriptional repression of lysine biosynthetic genes by lysine (Feller *et al.*, Eur. J. Biochem. 261: 163-70 (1999)), the decreased stability of both the mRNA encoding the uracil permease Fur4p and the permease itself in the presence of uracil (Seron *et* al., J. Bacteriol. 181: 1793-800 (1999)), and the inhibition of alpha-isopropyl malate synthase, a key step in leucine biosynthesis, by the presence of leucine (Beltzer *et al.*, J. Biol. Chem. 263: 368-74 (1988)).

Transporters that could mediate resistance to secondary metabolites include members of the major facilitator superfamily (MFS) and the ATP binding cassette (ABC) transporters. For example, overexpression of the class I MFS-type transporter Flr1p in *S. cerevisiae* has been shown to confer resistance to a variety of toxic compounds such as cycloheximide, fluconazole, 4-nitroquinolone oxide, and cerulenin (Alarco *et* al., J. Biol. Chem. 272: 19304-13 (1997); Oskouian and Saba, Mol. Gen. Genet. 261: 346-53 (1999)). MFS transporters have been functionally grouped into 23 families in yeast, several of which contain members known or suspected to mediate resistance to toxic compounds by promoting their efflux from the cell (reviewed by Nelissen *et al.* in FEMS Microbiol. Rev. 21: 113-34 (1997)). Likewise, ABC transporters encoded by genes including *PDR5* from *S. cerevisiae* (Boyum and Guidotti, Biochem. Biophys. Res. Commun. 230: 22-6 (1997)), *PMR1* from *Penicillium digitatum* (Nakuane *et al.*, Appl. Environ. Microbiol. 64: 3983-8 (1998)) and *MDR1* from *Candida albicans* (Sanglard *et al.*, Antimicrob. Agents Chemother. 39: 2378-86 (1995)), amongst others, have been shown to confer resistance to a variety of toxic compounds when their expression is increased. A complete cataloging of ABC transporters in yeast, as well as predicted function based on sequence similarities to transporters of known function, is described in (Decottignies and Goffeau, Nat. Genet. 15: 137-45 (1997)).

Transcription factors that regulate the expression of efflux pumps could also be used to increase efflux of a drug from a fungal cell to increase yields of a metabolite and decrease toxicity of the secondary metabolite in a fermentation. Such transcription factors include, but are not limited to, genes such as *YAP1*, *PDR1*, and *PDR3* from *S. cerevisiae* and their homologs. Overexpression of each of these genes has been shown to upregulate expression of transporters and cause increased resistance of *S. cerevisiae* to toxic compounds (for examples, see Reid *et al.*, J. Biol. Chem. 272: 12091-9 (1997); Katzmann *et al.*, Mol. Cell. Biol. 14: 4653-61 (1994); Wendler *et al.*, J. Biol. Chem. 272: 27091-8 (1997)).

Resistance to the toxic effects of secondary metabolites mediated through modulating expression of target genes will vary with metabolite. For example, amatoxins kill cells by inhibiting the function of the major cellular RNA polymerase, RNA polymerase II, in eucaryotic cells. Mutant forms of RNA polymerase II resistant to the effects of alpha-amanitin have been described (Bartolomei *et al.*, Mol. Cell. Biol. 8: 330-9 (1988); Chen *et al.*, Mol. Cell. Biol. 13: 4214-22 (1993)). Similarly, mutations affecting HMG CoA reductase, the target enzyme for the secondary metabolite lovastatin, have been identified. Increased levels of HMG CoA Reductase can also cause resistance to lovastatin (Ravid *et al.*, J. Biol. Chem. 274: 29341-51 (1999); Lum *et al.*, Yeast 12: 1107-24 (1996)). Taxol (paclitaxel), causes lethality by increasing microtubule stability, thus preventing exit from mitosis. Dominant mutations affecting beta-tubulin that confer resistance to taxol have been characterized (for example, see Gonzalez *et al.*, J. Biol. Chem. 274: 23875-82 (1999)) and could prove to be useful to confer resistance of production strains to this toxic metabolite. Such mutations appear to decrease the stability of microtubules; whether these mutations affect the binding of taxol to microtubules is not known. Similarly, modulating expression of other genes that decrease the stability of microtubules could also confer taxol resistance to a fungus that produces taxol. The pneumocandin and echinocandin families of metabolites are fungal secondary metabolites that inhibit the enzyme 1,3-beta-D-glucan synthase. Dominant mutations in the *Candida albicans* glucan synthase gene, *FKS1*, have been shown to confer resistance to candins (Douglas *et al.*, Antimicrob. Agents Chemother. 41: 2471-9 (1997)). Glucan synthase mutations such as these could be used to generate fungal production strains with increased resistance to the candin class of antifungals. *S. cerevisiae* mutants resistant to the growth-inhibitory effects of the fungal secondary metabolite cyclosporin A have also been

described (Cardenas *et al.*, EMBO J 14: 2772-83 (1995)). These mutants were shown to harbor mutations in *CNA1*, the gene encoding the catalytic subunit of the heterodimeric calcium-calmodulin dependent phosphatase, calcineurin A. Fumagillin, an antiangiogenic agent, binds to and inhibits the N-terminal aminopeptidases in a wide variety of both procaryotes and eucaryotes (Sin *et al.*, Proc. Natl. Acad. Sci. USA 94: 6099-103 (1997), Lowther *et al.*, Proc. Natl. Acad. Sci. USA 95: 12153-7 (1998)). Mutations in this enzyme that block fumagillin binding and/or inhibitory activity could well prove useful in enhancing the resistance of fungal production strains to the growth inhibitory effects of this secondary metabolite.

To demonstrate the feasibility of engineering a fungal strain to be resistant to otherwise toxic amounts of a secondary metabolite, two genes from the lovastatin biosynthetic cluster of *A. terreus* strain ATCC 20542 were used (Kennedy *et al.*, Science. 284: 1368-72 (1999)). These genes are predicted to encode proteins, denoted by Genbank accession numbers AAD34558 (hereafter referred to as PUMP1) and AAD34564 (hereby referred to as PUMP2), that are members of the MFS class of transporters. As described above, some MFS transporters are known to confer resistance to toxic compounds. PUMP1 and PUMP2 were tested for their ability to confer resistance to otherwise toxic levels of lovastatin when expressed in the fungus *S. cerevisiae*.

Aspergillus terreus (MF22; ATCC#20542) was grown for 45 hours in Production Media at 25°C (Production Media contains Cerelose, 4.5% (w/v) Peptonized Milk, 2.5% (w/v) Autolyzed yeast, 0.25% (w/v) Polyglycol P2000, 0.25% (w/v) pH to 7.0). Mycelia were harvested in a 50cc syringe plugged with sterile cotton wool using a vacuum apparatus, washed once with sterile H2O, and snap frozen in liquid nitrogen. Mycelia were then ground to a powder under liquid nitrogen in a mortar and pestle, and homogenized in RLC buffer (Qiagen RNeasy Kit; Qiagen Inc., 28159 Avenue Stanford, Valencia CA 93155) using a GLH rotor-stator homogenizer (Omni International, 6530 Commerce Ct., Suite 100, Warrenton, VA 20817.) Total RNA was purified using a RNeasy Maxi column according to the instructions of the manufacturer.

The polyA+ fraction of the *A. terreus* total RNA was isolated using Oligotex beads (Qiagen Inc.). Purified polyA+ RNA (5 µg) was used to generate complementary DNA (cDNA)

using Superscript Reverse Transcriptase (Gibco BRL, 9800 Medical Center Drive, PO Box 6482, Rockville, MD 20849) according to the instructions of the manufacturer.

The cDNA was then used to isolate and clone PUMP1 and PUMP2 gene sequences using the polymerase chain reaction (PCR) and Gateway (Life Technologies) Cloning Technology (US Patent 5,888,732). Oligonucleotide sequences used for PCR were 5'-ACAAAAAAGCAGGCTCCACAATGACATCCCACCACGGTGA-3' (SEQ ID NO: 7) and 5'-ACAAGAAAGCTGGGTTCATTCGCTCCGTCCTTTCT-3' (SEQ ID NO: 8) for PUMP1. Oligonucleotide sequences used for PUMP2 PCR were 5'-ACAAAAAAGCAGGCTCCACAATGGGCCGCGGTGACACTGA-3' (SEQ ID NO: 9) and 5'-ACAAGAAAGCTGGGTCTATTGGGTAGGCAGGTTGA-3' (SEQ ID NO: 10). The resultant plasmids, MB1333 and MB1334, were designed to express PUMP1 and PUMP2, respectively, under control of the *S. cerevisiae* promoter TEF1. The plasmids carry a functional *URA3* gene to allow for selection of the plasmid on media lacking uracil in a *ura3* mutant strain. These plasmids also contained a 2-micron origin for high-copy replication in yeast. Control plasmids were as follows: MB969, the parent vector for MB1333 and MB1334, that does not contain a heterologous gene and is not expected to confer resistance to a yeast strain; MB1344, constructed and described in Donald *et al.*, Appl. Environ. Microbiol. 63: 3341-4 (1997) as pRH127-3, that expresses a soluble form of HMG CoA reductase under control of the yeast *GPD1* promoter and is known to confer resistance to increased levels of lovastatin (Donald *et al.*, Appl. Environ. Microbiol. 63: 3341-4 (1997)).

MB1333, MB1334, MB969 and MB1344 were transformed into the yeast strain 22409 (Research Genetics, USA) using standard transformation methods for *S. cerevisiae* (Biotechniques, 1992, 13(1): 18). Strain 22409 is derived from the S288c strain background of *S. cerevisiae*, and its complete genotype is as follows: *MATa/α, his3Δ1/his3Δ1, leu2Δ0/leu2Δ0, ura3Δ0/ura3Δ0, LYS2/lys2Δ0, MET15/met15Δ0 pdr5::G418/PDR5.* Transformants were grown overnight at 30°C in synthetic complete media lacking uracil (SC-U) to maintain selection for the plasmid. Cultures were diluted 1:10 in sterile water, and 5μl of each strain was spotted to SC-URA agar containing different concentrations of lovastatin as shown in Figure 1. Strikingly, the strain harboring MB1333, and thus expressing PUMP1, shows resistance to lovastatin equivalent to the positive control strain in which the soluble fragment of HMG CoA reductase is

overexpressed (strain carrying MB1344). These strains show no obvious growth inhibition even at the highest concentrations of lovastatin tested (150µg/ml). In contrast, the vector-only control and the strain expressing PUMP2 show growth inhibition at the lowest concentration of lovastatin tested (50 µg/mL). Thus, these data indicate that PUMP1 is an excellent candidate for use in engineering lovastatin producing strains to enhance resistance to lovastatin and to promote efflux of this secondary metabolite.

Example 6

Causing conditional lysis of a fungus

Methods for improving the production of secondary metabolites can involve the construction of strains with desired characteristics for growth or recovery of secondary metabolites. Optimal strain characteristics likely will vary depending upon the fungus being utilized, the particular secondary metabolite being produced, and the specifications of an individual fermentation apparatus. Two traits that might be advantageous for maximal production of secondary metabolites are strains that can be lysed under specific conditions and strains that have morphological characteristics such as increased surface area of active growth and decreased hyphal length. Described below are examples of how both of these traits can be affected by modulating the activity of small GTP-binding proteins (G-proteins).

Fungi must respond to adverse external signals such as osmotic stress. Media for production of secondary metabolites often are hypo-osmotic, whereas fungi that exist on desiccated surfaces must respond to hyper-osmotic stress. One response to hyper-osmotic conditions is to increase the intracellular concentration of osmolytes such as glycerol. During hypo-osmotic stress the integrity of a fungal cell can be maintained both by decreasing intracellular osmolyte concentrations as well as by cell wall modifications. In *Saccharomyces cerevisiae* the *PKC1-SLT2* signaling pathway is required for growth in conditions of low osmolarity (reviewed in Heinisch *et al.*, Mol. Microbiol. 32: 671-680 (1999)). *PKC1*, which encodes yeast protein kinase C, is activated by components such as the small GTP-binding protein Rho1. Pkc1 then transduces this signal to a MAP kinase signaling cascade that includes

the MEK kinase Bck1, the functionally redundant MEKs Mkk1 and Mkk2, and the MAP kinase Slt2. Mutations in genes encoding these signaling components result in varying degrees of cell lysis on media of low osmolarity. Genetic screens have identified many other proteins that function either upstream of *PKC1-SLT2* signaling or regulate specific pathway components. These factors include Ppz1, Ppz2, Pph21, Pph22, Ptp2, Ptp3, Isr1, Rom1, Rom2, Mid2, Slg1, Wsc2, Wsc3, Wsc4, Stt3, Stt4, and Vps45; many of these components have homologs in other fungi. In addition, transcription factors, such as Rlm1, Swi4, and Swi6, that can function downstream of *PKC1-SLT2* signaling have been identified, and it has been demonstrated that some of these factors are required for the proper expression of genes involved in cell wall biosynthesis. Thus, many components that can modulate the structural integrity of yeast cells have been identified. It is possible that manipulation of these factors could be performed, such that conditional expression of variants of these genes (or the homologs from filamentous fungi) would result in the lysis of fungi and maximal recovery of secondary metabolites.

Conditional lysis of fungi at the conclusion of a fermentor run would be a powerful method for promoting increased recovery of secondary metabolite. Preferably, conditional lysis would require a simple manipulation such as a change in a standard growth parameter (*e.g.* temperature, dissolved oxygen) or addition of an inexpensive solute. Examples of small molecules that may cause cell lysis include the protein kinase C inhibitor staurosporine, caffeine, dyes that bind the cell wall polymer chitin (*e.g.* calcofluor white, Congo red), inhibitors of glucan synthase (*e.g.* candins), and inhibitors of chitin synthase. The cost of using these molecules in a large-scale fermentor likely would be prohibitive. Similarly, addition of enzymes such as glucanases or chitinases would likely be an effective, but costly, method for inducing lysis. An alternative means to induce lysis would be the conditional expression of a dominant negative mutation in a gene encoding a component required for cell wall integrity. Since many components of the *PKC1-SLT2* signaling pathway are widely conserved, it is possible that the conditional expression of a dominant inhibitory form of a member of this pathway would facilitate lysis in a variety of fungi, including those fungi that produce secondary metabolites such as lovastatin and cyclosporin A.

The G-protein Rho1 functions to regulate cell wall integrity by at least two independent mechanisms; Rho1 activates Pkc1 signaling as well as 1,3-beta-glucan synthase activity (Nonaka

et al., EMBO J. 14: 5931-5938 (1995); Drgonova *et al.*, Science 272: 277-279 (1996); Qadota *et al.*, Science 272: 279-281 (1996)). In addition, dominant inhibitory forms of Rho1 have been identified. Expression of a *rho1G22S D125N* mutant form in a wild-type *Saccharomyces cerevisiae* strain results in cell lysis. Therefore, the conditional expression of dominant inhibitory forms of Rho1 under the control of a heat-shock inducible promoter might be an effective method for causing cell lysis in production fungi.

RHO1 coding sequence for construction of dominant mutations can be isolated from *Saccharomyces cerevisiae* genomic DNA. Primers 5'-cgc**GGATCC**CGACATATTCGAGGTTGACT-3' (SEQ ID NO: 11) and 5'-ccc**AAGCTT**GCTAGAAATATGAACCTTCC-3' (SEQ ID NO: 12) are used to amplify *RHO1* coding sequence with 1 kilobase of upstream regulatory sequence and 500 basepairs of downstream regulatory sequence. *Bam*HI and *Hind*III restriction sites are added to the oligonucleotides to facilitate cloning into the pRS416 centromere-based yeast vector. The Quik Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla CA) is used to first create a mutation that encodes the G22S substitution; next, the pRS416*rho1G22S* plasmid is used as a template to introduce a mutation that encodes the D125N substitution. Primer pair 5'-gtgcctgt**A**gtaagacatgt-3' / 5'-acatgtcttac**T**acaggcac-3' is used to anneal to the pRS416*RHO1* template for pRS416*rho1G22S* allele construction. Primer pair 5'-gtaaagtg**A**atttgagaaac-3' / 5'-gtttctcaaat**T**cactttac-3' is used to anneal to the pRS416*rho1G22S* template for pRS416*rho1G22S D125N* allele construction. pRS416*rho1G22S D125N* and control plasmids (pRS416*RHO1* and pRS416) are then used to transform a wild-type *ura3* auxotrophic strain. Transformants are selected and grown at 25°C in synthetic liquid growth medium lacking uracil and containing the osmolyte sorbitol (1M). Cultures are then transferred to growth in synthetic liquid growth medium lacking uracil without sorbitol, and cells are visually inspected following growth for various periods of time. Expression of the *rho1G22S D125N* dominant allele causes cell lysis after growth for approximately 120 minutes.

Conditional promoters can be used to express *RHO1* dominant mutations in filamentous fungi. The *Aspergillus niger tpsB* gene is expressed at low levels during growth at ambient temperatures, whereas expression is strongly enhanced upon heat-shock at 40°C; *tpsB* regulatory sequence contains multiple copies of the CCCCT stress responsive element (Wolschek *et al.*, J.

Biol. Chem. 272: 2729-2735 (1997)). Primers 5'-catg**GGGCCC**TCTCTCCACCGGCACTAAGATAGC-3' (SEQ ID NO: 13) and 5'-cgc**GGATCC**ag**CAT**TGGAAAAGGAGGGGGGGGAAG-3' (SEQ ID NO: 14) are used to amplify 490 basepairs of *tpsB* upstream regulatory sequence from *A. niger* genomic DNA. This PCR product contains the *tpsB* start codon followed by a *Bam*HI cloning site. The *tpsB* upstream regulatory sequence can be cloned as an *ApaI/Bam*HI fragment into the filamentous fungal vector pLXZ116 (see Example 1). The *tpsB* promoter is cloned into a multiple cloning site that also contains terminator sequence of the *A. nidulans trpC* gene. Primers 5'-cgc**GGATCC**aTCACAACAAGTTGGTAACAGTATC-3' (SEQ ID NO: 15) and 5'-gg**ACTAGT**TAACAAGACACACTTCTTCTTCTT-3' (SEQ ID NO: 16) are used to amplify *rho1G22S D125N* coding sequence, and the product is cloned into the *Bam*HI/*Spe*I sites of the *tpsB* containing filamentous fungal vector. This vector can be used to conditionally express (at 40°C) a dominant negative form of Rho1 that can cause cell lysis.

The filamentous fungal vector containing the *tpsB* promoter (no *RHO1* insert) and a vector containing *rho1G22S D125N* are used to transform *Aspergillus nidulans*, *Penicillium chrysogenum*, and *Aspergillus terreus*. To assess the impact of conditional expression of a *RHO1* dominant negative mutation on cell wall integrity of filamentous fungi, mycelia or spore preps are made from 10 independent PCR-positive transformants, and mycelia or spores are used to inoculate both liquid shake flask cultures and plates containing minimal or rich medium. After growth for 1-2 days the strains are transferred to both 37°C and 40°C. Strains are examined for morphological defects over the next 24 hours of incubation; potential morphological defects include abnormalities in polarized growth, hyphal wall integrity, and conidiophore development. The optimal time of heat-shock induction required for lysis will be determined. Furthermore, it will be determined whether any abnormalities can be suppressed by growth on medium containing osmotic stabilizers such as sorbitol (1.2 M), sucrose (1 M), or NaCl (1.5 M).

Transformants of *Aspergillus terreus* that display morphological abnormalities are used to assess whether conditional lysis of strains can be a tool for recovering larger quantities of lovastatin from fermentation broths. Five independent PCR-positive *RHO1*-containing transformants that display lysis defects will be processed as the *A. terreus* transformants

described in earlier examples. Cultures from each transformant and control strains will be grown for either 8, 9, 10, 11, or 12 days, and cultures will then be incubated at the optimal temperature and for the optimal time required for cell lysis. Following heat shock the cell mass from each culture is separated from the broth by filtration, and the cell mass is lyophilized and weighed. Lovastatin concentration in the broth is calculated as described in earlier examples.

Morphological characteristics such as decreased hyphal length might be advantageous during production of secondary metabolites. For example, strains with shorter filament lengths should display decreased entanglement, floc formation, and shear stress. Such strains would be less susceptible to shear stress damage, these strains might reduce viscosity and facilitate mass transfer, and short filament strains might save energy costs required to power impellers. Increasing the amount of hyphal branching should result in an overall decrease in filament length. The following example describes how expression of a dominant inactive form of the *Saccharomyces cerevisiae* Rsr1 protein (also known as Bud1) results in increased lateral branch formation.

The yeast Rsr1 protein is required for proper bud site selection; strains lacking Rsr1 bud at random sites on the cell surface. Dominant negative mutations such as *rsr1K16N* have been identified, and expression of these mutant forms cause random bud site selection without causing obvious growth defects. Expression of *rsr1K16N* in filamentous fungi may increase branching, decrease filament length, and not have deleterious effects on the growth of the organism.

RSR1 coding sequence for construction of dominant mutations can be isolated from *Saccharomyces cerevisiae* genomic DNA. Primers 5'-cgc**GGATCC**TATCTTCACTCAATATACTTCCTA-3' (SEQ ID NO: 17) and 5'-ccc**AAGCTT**CATCGTTGAAACTTGATAACGCAC-3' (SEQ ID NO: 18) are used to amplify *RHO1* coding sequence with 750 basepairs of upstream regulatory sequence and 500 basepairs of downstream regulatory sequence. *Bam*HI and *Hind*III restriction sites are added to the oligonucleotides to facilitate cloning into the pRS416 centromere-based yeast vector. The Quik Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla CA) is used to create dominant-negative *RSR1* substitution mutation K16N. Primer pairs 5'- tggtgtcggtaaTtcctgcttaac-3' / 5'-gttaagcaggaAttaccgacacca-3' is used to anneal to the pRS416*RSR1* template for allele construction. The pRS416*rsr1K16N* and control pRS416 plasmids are then used to transform a

haploid wild-type *ura3* auxotrophic strain. Transformants are selected and grown at 30°C in YPD liquid growth medium. Log phase cultures are fixed in 3.7% formaldehyde (vol:vol) and stained with the chitin-binding dye Calcofluor white, as described; previous sites of bud formation are marked with a chitin-rich structure called a bud scar. Fluorescent microscopy reveals that cells containing the control plasmid display clustering of bud scars at one pole of the cells, the well-characterized haploid pattern of bud site selection. Cells expressing *rsr1K16N* display a random pattern of bud site selection; bud scars are scattered across the surface of haploid cells. Cells expressing *rsr1K16N* do not display other obvious growth or morphological defects.

The *Aspergillus nidulans* PGK promoter can be used to express *RSR1* dominant mutations in filamentous fungi. A filamentous fungal vector containing a multiple cloning site that is flanked by the PGK promoter and terminator sequence of the *A. nidulans trpC* gene is used. Primers 5'-cgc**GGATCC**GACTAATGAGAGACTATAAATTAG-3' (SEQ ID NO: 19) and 5'-ccg**CTCGAG**CTATAGAATAGTGCAAGTGGAAGC-3' (SEQ ID NO: 20) are used to amplify *rsr1K16N* coding sequence, and the product is cloned into the *Bam*HI/*Xho*I sites of the filamentous fungal vector. This vector can be used to express a dominant negative form of Rsr1 that will affect the process of selecting sites for polarized growth.

The filamentous fungal vector containing *rsr1K16N* and control vector are used to transform *Aspergillus nidulans*, *Penicillium chrysogenum*, and *Aspergillus terreus*. To assess the impact of expression of *RSR1* dominant negative mutations on lateral branch formation and filament length, mycelia and spore preps are made from 10 independent PCR-positive transformants, and mycelia and spores are used to inoculate both liquid shake flask cultures and plates containing minimal or rich medium. Strains are examined at various timepoints over a 48 hour period for morphological alterations, including altered patterns of germ tube emergence, increased lateral branching, decreased filament length, alterations in hyphal width, and changes in chitin staining pattern. Strains displaying desirable morphological changes are then tested in shake flask conditions to determine whether levels of penicillin (*A. nidulans*, *P. chrysogenum*) or lovastatin (*A. terreus*) production have changed significantly.

Aspergillus terreus and *Penicillium chrysogenum* transformants that display morphological characteristics such as decreased filament length and produce expected or greater

levels of lovastatin and penicillin, respectively, are used to assess whether morphological changes can impact upon bioreactor challenges such as shear stress damage, mass transfer, and energy costs. Five independent PCR-positive *RSR1*-containing transformants that display morphological alterations are grown in a small-scale bioreactor, and examined for improved fermentation characteristics and/or production of secondary metabolite.

What is claimed is:

1. A method for improving production of a secondary metabolite by a fungus by increasing the yield of the secondary metabolite in the fungus, the method comprising modulating the expression of a gene involved in regulation of secondary metabolite production in a manner that improves the yield of the secondary metabolite, provided however, that when the secondary metabolite is isopenicillin N, then the modulation is not mediated by transcription factor CPCR1, and when the gene involved in regulation of secondary metabolite production is from *Saccharomyces cerevisiae*, then the modulation is not through decreased activity or expression of HOG1, BEM2, RIM15, SFL1, IRA1, SSD1, SRB11, SWI4, TPK3 or though increased activity or expression of AFL1, DHH1, INV7, INV8, STE21, PET9, MEP2, INV1, INV5, INV6, INV9, INV10, INV11, INV12, INV13, INV14, INV15, CDC25, MCM1, MGA1, PHD2, PHO23, PTC1, RIM1, STP22, TPK2 or YPR1.

2. The method according to claim 1, wherein the modulation is overexpression of the gene.

3. The method according to claim 1, wherein the modulation is conditional expression of the gene.

4. The method according to claim 1, wherein the modulation is expression of a dominant mutation of the gene.

5. The method according to claim 4, wherein the dominant mutation is a dominant negative mutation.

6. The method according to claim 4, wherein the dominant mutation is a dominant positive mutation.

7. The method according to claim 4, wherein the dominant mutation is a dominant neomorphic mutation.

8. The method according to claim 1, wherein the modulation is mediated by a transcription factor.

9. The method according to claim 1, wherein the modulation is mediated by a peptide modulator of gene expression.

10. The method according to claim 9, wherein the peptide modulator is an activator of gene expression.

11. The method according to claim 9, wherein the peptide modulator is an inhibitor of gene expression.

12. The method according to claim 1, wherein the modulation is mediated by a small molecule modulator of gene expression.

13. The method according to claim 12, wherein the small molecule modulator is an activator of gene expression.

14. The method according to claim 12, wherein the small molecule modulator is an inhibitor of gene expression.

15. The method according to any of claims 1-14, wherein the gene acts on a transcription factor.

16. The method according to any of claims 1-14, wherein the gene acts on a transmembrane transporter.

17. The method according to claim 16, wherein the transmembrane transporter is a pump.

18. The method according to any of claims 1-14, wherein the gene acts on a kinase.

19. The method according to any of claims 1-14, wherein the gene acts on a G-protein.

20. The method according to any of claims 1-14, wherein the gene acts on a phosphatase.

21. The method according to any of claims 1-14, wherein the gene acts on a protease.

22. The method according to any of claims 1-14, wherein the gene acts on a biosynthetic enzyme.

23. The method according to any of claims 1-14, wherein the gene acts on a cell surface receptor.

24. The method according to any of claims 1-14, wherein the gene acts on a GTPase activating protein.

25. The method according to any of claims 1-14, wherein the gene acts on a guanine nucleotide exchange factor.

26. The method according to any of claims 1-14, wherein the gene acts on a cyclic nucleotide phosphodiesterase.

27. The method according to any of claims 1-14, wherein the gene acts on a bacterial protein toxin.

28. The method according to any of claims 1-14, wherein the gene acts on an importin protein.

29. The method according to any of claims 1-14, wherein the gene acts on an RNA binding protein.

30. The method according to any of claims 1-14, wherein the gene acts on a component of a SCF complex.

31. The method according to any of claims 1-14, wherein the gene encodes a transcription factor.

32. The method according to any of claims 1-14, wherein the gene encodes a transmembrane transporter.

33. The method according to claim 32, wherein the transmembrane transporter is a pump.

34. The method according to any of claims 1-14, wherein the gene encodes a kinase.

35. The method according to any of claims 1-14, wherein the gene encodes a G-protein.

36. The method according to any of claims 1-14, wherein the gene encodes a phosphatase.

37. The method according to any of claims 1-14, wherein the gene encodes a protease.

38. The method according to any of claims 1-14, wherein the gene encodes a biosynthetic enzyme.

39. The method according to any of claims 1-14, wherein the gene encodes a cell surface receptor.

40. The method according to any of claims 1-14, wherein the gene encodes a GTPase activating protein.

41. The method according to any of claims 1-14, wherein the gene encodes a guanine nucleotide exchange factor.

42. The method according to any of claims 1-14, wherein the gene encodes a cyclic nucleotide phosphodiesterase.

43. The method according to any of claims 1-14, wherein the gene encodes a bacterial protein toxin.

44. The method according to any of claims 1-14, wherein the gene encodes an importin protein.

45. The method according to any of claims 1-14, wherein the gene encodes an RNA binding protein.

46. The method according to any of claims 1-14, wherein the gene encodes a component of a SCF complex.

47. The method according to any of claims 1-19, wherein the secondary metabolite is an antibacterial.

48. The method according to claim 20, wherein the antibacterial is a ß-lactam.

49. The method according to claim 20, wherein the antibacterial is a cephalosporin.

50. The method according to any of claims 1-19, wherein the secondary metabolite is an anti-hypercholesterolemic.

51. The method according to claim 23, wherein the anti-hypercholesterolemic is selected from lovastatin, mevastatin, simvastatin and pravastatin.

52. The method according to any of claims 1-46, wherein the secondary metabolite is an immunosuppressant.

53. The method according to claim 25, wherein the immunosuppressant is a cyclosporin.

54. The method according to any of claims 1-46, wherein the secondary metabolite is an ergot alkaloid.

55. The method according to any of claims 1-46, wherein the secondary metabolite is an angiogenesis inhibitor.

56. The method according to any of claims 1-46, wherein the secondary metabolite is a glucan synthesis inhibitor.

57. The method according to any of claims 1-46, wherein the secondary metabolite is a fungal toxin.

58. The method according to any of claims 1-46, wherein the secondary metabolite is a gliotoxin.

59. The method according to any of claims 1-46, wherein the secondary metabolite is a modulator of cell surface receptor signaling.

60. The method according to any of claims 1-46, wherein the secondary metabolite is a plant growth regulator.

61. The method according to any of claims 1-46, wherein the secondary metabolite is a pigment.

62. The method according to any of claims 1-46, wherein the secondary metabolite is an insecticide.

63. The method according to any of claims 1-46, wherein the secondary metabolite is an anti-neoplastic compound.

64. The method according to any of claims 1-63, further comprising the step of purifying the secondary metabolite from a culture of the fungus.

65. A method for improving production of a secondary metabolite by a fungus by increasing productivity of the secondary metabolite in the fungus, the method comprising modulating the expression of a gene involved in regulation of secondary metabolite production in a manner that improves the productivity of the secondary metabolite, provided however, that when the secondary metabolite is isopenicillin N, then the modulation is not mediated by transcription factor CPCR1, and when the gene involved in regulation of secondary metabolite production is from *Saccharomyces cerevisiae*, then the modulation is not through decreased activity or expression of HOG1, BEM2, RIM15, SFL1, IRA1, SSD1, SRB11, SWI4, TPK3 or though increased activity or expression of AFL1, DHH1, INV7, INV8, STE21, PET9, MEP2, INV1, INV5, INV6, INV9, INV10, INV11, INV12, INV13, INV14, INV15, CDC25, MCM1, MGA1, PHD2, PHO23, PTC1, RIM1, STP22, TPK2, YPR1, or HAP4.

66. The method according to claim 65, wherein the modulation is overexpression of the gene.

67. The method according to claim 65, wherein the modulation is conditional expression of the gene.

68. The method according to claim 65, wherein the modulation is expression of a dominant mutation of the gene.

69. The method according to claim 68, wherein the dominant mutation is a dominant negative mutation.

70. The method according to claim 68, wherein the dominant mutation is a dominant neomorphic mutation.

71. The method according to claim 68, wherein the dominant mutation is a dominant positive mutation.

72. The method according to claim 65, wherein the modulation is mediated by a transcription factor.

73. The method according to claim 65, wherein the modulation is mediated by a peptide modulator of gene expression.

74. The method according to claim 73, wherein the peptide modulator is an activator of gene expression.

75. The method according to claim 73, wherein the peptide modulator is an inhibitor of gene expression.

76. The method according to claim 65, wherein the modulation is mediated by a small molecule modulator of gene expression.

77. The method according to claim 76, wherein the small molecule modulator is an activator of gene expression.

78. The method according to claim 76, wherein the small molecule modulator is an inhibitor of gene expression.

79. The method according to any of claims 65-78, wherein the gene acts on a transcription factor.

80. The method according to any of claims 65-78, wherein the gene acts on a transmembrane transporter.

81. The method according to any of claims 65-78, wherein the gene acts on a kinase.

82. The method according to any of claims 65-78, wherein the gene acts on a G-protein.

83. The method according to any of claims 65-78, wherein the gene acts on a phosphatase.

84. The method according to any of claims 65-78, wherein the gene acts on a protease.

85. The method according to any of claims 65-78, wherein the gene acts on a biosynthetic enzyme.

86. The method according to any of claims 65-78, wherein the gene acts on a cell surface receptor.

87. The method according to any of claims 65-86, wherein the gene acts on a GTPase activating protein.

88. The method according to any of claims 65-86, wherein the gene acts on a guanine nucleotide exchange factor.

89. The method according to any of claims 65-86, wherein the gene acts on a cyclic nucleotide phosphodiesterase.

90. The method according to any of claims 65-86, wherein the gene acts on a bacterial protein toxin.

91. The method according to any of claims 65-86, wherein the gene acts on an importin protein.

92. The method according to any of claims 65-86, wherein the gene acts on an RNA binding protein.

93. The method according to any of claims 65-86, wherein the gene acts on a component of a SCF complex.

94. The method according to any of claims 65-86, wherein the gene encodes a transcription factor.

95. The method according to any of claims 65-86, wherein the gene encodes a transmembrane transporter.

96. The method according to claim 95, wherein the transmembrane transporter is a pump.

97. The method according to any of claims 65-86, wherein the gene encodes a kinase.

98. The method according to any of claims 65-86, wherein the gene encodes a G-protein.

99. The method according to any of claims 65-86, wherein the gene encodes a phosphatase.

100. The method according to any of claims 65-86, wherein the gene encodes a protease.

101. The method according to any of claims 65-86, wherein the gene encodes a biosynthetic enzyme.

102. The method according to any of claims 65-86, wherein the gene encodes a cell surface receptor.

103. The method according to any of claims 65-86, wherein the gene encodes a GTPase activating protein.

104. The method according to any of claims 65-86, wherein the gene encodes a guanine nucleotide exchange factor.

105. The method according to any of claims 65-86, wherein the gene encodes a cyclic nucleotide phosphodiesterase.

106. The method according to any of claims 65-86, wherein the gene encodes a bacterial protein toxin.

107. The method according to any of claims 65-86, wherein the gene encodes an importin protein.

108. The method according to any of claims 65-86, wherein the gene encodes an RNA binding protein.

109. The method according to any of claims 65-86, wherein the gene encodes a component of a SCF complex.

110. The method according to any of claims 65-109, wherein the secondary metabolite is an antibacterial.

111. The method according to claim 110, wherein the antibacterial is a ß-lactam.

112. The method according to claim 110, wherein the antibacterial is a cephalosporin.

113. The method according to any of claims 65-109, wherein the secondary metabolite is an anti-hypercholesterolemic.

114. The method according to claim 113, wherein the anti-hypercholesterolemic is selected from lovastatin, mevastatin, simvastatin and pravastatin.

115. The method according to any of claims 65-109, wherein the secondary metabolite is an immunosuppressant.

116. The method according to claim 115, wherein the immunosuppressant is a cyclosporin.

117. The method according to any of claims 65-109, wherein the secondary metabolite is an ergot alkaloid.

118. The method according to any of claims 65-109, wherein the secondary metabolite is an angiogenesis inhibitor.

119. The method according to any of claims 65-109, wherein the secondary metabolite is a glucan synthesis inhibitor.

120. The method according to any of claims 65-109, wherein the secondary metabolite is a fungal toxin.

121. The method according to any of claims 65-109, wherein the secondary metabolite is a gliotoxin.

122. The method according to any of claims 65-109, wherein the secondary metabolite is a modulator of cell surface receptor signaling.

123. The method according to any of claims 65-109, wherein the secondary metabolite is a plant growth regulator.

124. The method according to any of claims 65-109, wherein the secondary metabolite is a pigment.

125. The method according to any of claims 65-109, wherein the secondary metabolite is an insecticide.

126. The method according to any of claims 65-109, wherein the secondary metabolite is an anti-neoplastic compound.

127. The method according to any of claims 65-126, further comprising the step of purifying the secondary metabolite from a culture of the fungus.

128. A method for improving production of a secondary metabolite in a fungus by increasing efflux or excretion of the secondary metabolite, the method comprising modulating the expression of a gene involved in regulation of secondary metabolite production in a manner that increases efflux or excretion the secondary metabolite.

129. The method according to claim 128, wherein the modulation is overexpression of the gene.

130. The method according to claim 128, wherein the modulation is conditional expression of the gene.

131. The method according to claim 128, wherein the modulation is expression of a dominant mutation of the gene.

132. The method according to claim 131, wherein the dominant mutation is a dominant negative mutation.

133. The method according to claim 131, wherein the dominant mutation is a dominant positive mutation.

134. The method according to claim 131, wherein the dominant mutation is a dominant neomorphic mutation.

135. The method according to claim 128, wherein the modulation is mediated by a transcription factor.

136. The method according to claim 128, wherein the modulation is mediated by a peptide modulator of gene expression.

137. The method according to claim 136, wherein the peptide modulator is an activator of gene expression.

138. The method according to claim 136, wherein the peptide modulator is an inhibitor of gene expression.

139. The method according to claim 128, wherein the modulation is mediated by a small molecule modulator of gene expression.

140. The method according to claim 139, wherein the small molecule modulator is an activator of gene expression.

141. The method according to claim 139, wherein the small molecule modulator is an inhibitor of gene expression.

142. The method according to any of claims 128-141, wherein the gene acts on a transcription factor.

143. The method according to any of claims 128-141, wherein the gene acts on a transmembrane transporter.

144. The method according to any of claims 128-141, wherein the gene acts on a kinase.

145. The method according to any of claims 128-141, wherein the gene acts on a G-protein.

146. The method according to any of claims 128-141, wherein the gene acts on a phosphatase.

147. The method according to any of claims 128-141, wherein the gene acts on a protease.

148. The method according to any of claims 128-141, wherein the gene acts on a biosynthetic enzyme.

149. The method according to any of claims 128-141, wherein the gene acts on a cell surface receptor.

150. The method according to any of claims 128-141, wherein the gene acts on a GTPase activating protein.

151. The method according to any of claims 128-141, wherein the gene acts on a guanine nucleotide exchange factor.

152. The method according to any of claims 128-141, wherein the gene acts on a cyclic nucleotide phosphodiesterase.

153. The method according to any of claims 128-141, wherein the gene acts on a bacterial protein toxin.

154. The method according to any of claims 128-141, wherein the gene acts on an importin protein.

155. The method according to any of claims 128-141, wherein the gene acts on an RNA binding protein.

156. The method according to any of claims 128-141, wherein the gene acts on a component of a SCF complex.

157. The method according to any of claims 128-141, wherein the gene encodes a transcription factor.

158. The method according to any of claims 128-141, wherein the gene encodes a transmembrane transporter.

159. The method according to any of claim 158, wherein the transmembrane transporter is a pump.

160. The method according to any of claims 128-141, wherein the gene encodes a kinase.

161. The method according to any of claims 128-141, wherein the gene encodes a G-protein.

162. The method according to any of claims 128-141, wherein the gene encodes a phosphatase.

163. The method according to any of claims 128-141, wherein the gene encodes a protease.

164. The method according to any of claims 128-141, wherein the gene encodes a biosynthetic enzyme.

165. The method according to any of claims 128-141, wherein the gene encodes a cell surface receptor.

166. The method according to any of claims 128-141, wherein the gene encodes a GTPase activating protein.

167. The method according to any of claims 128-141, wherein the gene encodes a guanine nucleotide exchange factor.

168. The method according to any of claims 128-141, wherein the gene encodes a cyclic nucleotide phosphodiesterase.

169. The method according to any of claims 128-141, wherein the gene encodes a bacterial protein toxin.

170. The method according to any of claims 128-141, wherein the gene encodes an importin protein.

171. The method according to any of claims 128-141, wherein the gene encodes an RNA binding protein.

172. The method according to any of claims 128-141, wherein the gene encodes a component of a SCF complex.

173. The method according to any of claims 128-172, wherein the secondary metabolite is an antibacterial.

174. The method according to claim 173, wherein the antibacterial is a ß-lactam.

175. The method according to claim 173, wherein the antibacterial is a cephalosporin.

176. The method according to any of claims 128-172, wherein the secondary metabolite is an anti-hypercholesterolemic.

177. The method according to claim 176, wherein the anti-hypercholesterolemic is selected from lovastatin, mevastatin, simvastatin and pravastatin.

178. The method according to any of claims 128-172, wherein the secondary metabolite is an immunosuppressant.

179. The method according to claim 178, wherein the immunosuppressant is a cyclosporin.

180. The method according to any of claims 128-172, wherein the secondary metabolite is an ergot alkaloid.

181. The method according to any of claims 128-172, wherein the secondary metabolite is an angiogenesis inhibitor.

182. The method according to any of claims 128-172, wherein the secondary metabolite is a glucan synthesis inhibitor.

183. The method according to any of claims 128-172, wherein the secondary metabolite is a fungal toxin.

184. The method according to any of claims 128-172, wherein the secondary metabolite is a gliotoxin.

185. The method according to any of claims 128-172, wherein the secondary metabolite is a modulator of cell surface receptor signaling.

186. The method according to any of claims 128-172, wherein the secondary metabolite is a plant growth regulator.

187. The method according to any of claims 128-172, wherein the secondary metabolite is a pigment.

188. The method according to any of claims 128-172, wherein the secondary metabolite is an insecticide.

189. The method according to any of claims 128-172, wherein the secondary metabolite is an anti-neoplastic compound.

190. The method according to any of claims 128-189, further comprising the step of purifying the secondary metabolite from a culture of the fungus.

191. A method for improving production of a secondary metabolite in a fungus by decreasing production of side products or competing secondary metabolites, the method comprising modulating the expression of a gene involved in regulation of secondary metabolite production in a manner that decreases production of side products or competing secondary metabolites.

192. The method according to claim 191, wherein the modulation is overexpression of the gene.

193. The method according to claim 191, wherein the modulation is conditional expression of the gene.

194. The method according to claim 191, wherein the modulation is expression of a dominant mutation of the gene.

195. The method according to claim 194, wherein the dominant mutation is a dominant negative mutation.

196. The method according to claim 194, wherein the dominant mutation is a dominant positive mutation.

197. The method according to claim 194, wherein the dominant mutation is a dominant neomorphic mutation.

198. The method according to claim 191, wherein the modulation is mediated by a transcription factor.

199. The method according to claim 191, wherein the modulation is mediated by a peptide modulator of gene expression.

200. The method according to claim 199, wherein the peptide modulator is an activator of gene expression.

201. The method according to claim 199, wherein the peptide modulator is an inhibitor of gene expression.

202. The method according to claim 191, wherein the modulation is mediated by a small molecule modulator of gene expression.

203. The method according to claim 202, wherein the small molecule modulator is an activator of gene expression.

204. The method according to claim 202, wherein the small molecule modulator is an inhibitor of gene expression.

205. The method according to any of claims 191-204, wherein the gene acts on a transcription factor.

206. The method according to any of claims 191-204, wherein the gene encodes a transmembrane transporter.

207. The method according to any of claims 191-204, wherein the gene acts on a kinase.

208. The method according to any of claims 85-96, wherein the gene acts on a G-protein.

209. The method according to any of claims 191-204, wherein the gene acts on a phosphatase.

210. The method according to any of claims 191-204, wherein the gene acts on a protease.

211. The method according to any of claims 191-204, wherein the gene acts on a biosynthetic enzyme.

212. The method according to any of claims 191-204, wherein the gene acts on a cell surface receptor.

213. The method according to any of claims 191-204, wherein the gene acts on a GTPase activating protein.

214. The method according to any of claims 191-204, wherein the gene acts on a guanine nucleotide exchange factor.

215. The method according to any of claims 191-204, wherein the gene acts on a cyclic nucleotide phosphodiesterase.

216. The method according to any of claims 191-204, wherein the gene acts on a bacterial protein toxin.

217. The method according to any of claims 191-204, wherein the gene acts on an importin protein.

218. The method according to any of claims 191-204, wherein the gene acts on an RNA binding protein.

219. The method according to any of claims 191-204, wherein the gene acts on a component of a SCF complex.

220. The method according to any of claims 191-204, wherein the gene encodes a transcription factor.

221. The method according to any of claims 191-204, wherein the gene encodes a transmembrane transporter.

222. The method according to claim 221, wherein the transmembrane transporter is a pump.

223. The method according to any of claims 191-204, wherein the gene encodes a kinase.

224. The method according to any of claims 191-204, wherein the gene encodes a G-protein.

225. The method according to any of claims 191-204, wherein the gene encodes a phosphatase.

226. The method according to any of claims 191-204, wherein the gene encodes a protease.

227. The method according to any of claims 191-204, wherein the gene encodes a biosynthetic enzyme.

228. The method according to any of claims 191-204, wherein the gene encodes a cell surface receptor.

229. The method according to any of claims 191-204, wherein the gene encodes a GTPase activating protein.

230. The method according to any of claims 191-204, wherein the gene encodes a guanine nucleotide exchange factor.

231. The method according to any of claims 191-204, wherein the gene encodes a cyclic nucleotide phosphodiesterase.

232. The method according to any of claims 191-204, wherein the gene encodes a bacterial protein toxin.

233. The method according to any of claims 191-204, wherein the gene encodes an importin protein.

234. The method according to any of claims 191-204, wherein the gene encodes an RNA binding protein.

235. The method according to any of claims 191-204, wherein the gene encodes a component of a SCF complex.

236. The method according to any of claims 191-235, wherein the secondary metabolite is an antibacterial.

237. The method according to claim 236, wherein the antibacterial is a ß-lactam.

238. The method according to claim 236, wherein the antibacterial is a cephalosporin.

239. The method according to any of claims 191-235, wherein the secondary metabolite is an anti-hypercholesterolemic.

240. The method according to claim 239, wherein the anti-hypercholesterolemic is selected from lovastatin, mevastatin, simvastatin and pravastatin.

241. The method according to any of claims 191-235, wherein the secondary metabolite is an immunosuppressant.

242. The method according to claim 241, wherein the immunosuppressant is a cyclosporin.

243. The method according to any of claims 191-235, wherein the secondary metabolite is an ergot alkaloid.

244. The method according to any of claims 191-235, wherein the secondary metabolite is an angiogenesis inhibitor.

245. The method according to any of claims 191-235, wherein the secondary metabolite is a glucan synthesis inhibitor.

246. The method according to any of claims 191-235, wherein the secondary metabolite is a fungal toxin.

247. The method according to any of claims 191-235, wherein the secondary metabolite is a gliotoxin.

248. The method according to any of claims 191-235, wherein the secondary metabolite is a modulator of cell surface receptor signaling.

249. The method according to any of claims 191-235, wherein the secondary metabolite is a plant growth regulator.

250. The method according to any of claims 191-235, wherein the secondary metabolite is a pigment.

251. The method according to any of claims 191-235, wherein the secondary metabolite is an insecticide.

252. The method according to any of claims 191-235, wherein the secondary metabolite is an anti-neoplastic compound.

253. The method according to any of claims 191-252, further comprising the step of purifying the secondary metabolite from a culture of the fungus.

254. A method for improving production of a secondary metabolite in a fungus by altering the characteristics of the fungus in a manner that is beneficial to the production of the secondary metabolite, the method comprising modulating the expression of a gene involved in regulation of secondary metabolite production in a manner that alters the characteristics of the fungus.

255. The method according to claim 254, wherein the altered characteristic is transition from hyphal growth to yeast form.

256. The method according to claim 254, wherein the altered characteristic is an increase or decrease in flocculence.

257. The method according to claim 254, wherein the altered characteristic is increased or decreased adhesion to a surface.

258. The method according to any of claims 254-257, wherein the modulation is overexpression of the gene.

259. The method according to any of claims 254-257, wherein the modulation is conditional expression of the gene.

260. The method according to any of claims 254-257, wherein the modulation is expression of a dominant mutation of the gene.

261. The method according to claim 260, wherein the dominant mutation is a dominant negative mutation.

262. The method according to claim 260, wherein the dominant mutation is a dominant positive mutation.

263. The method according to claim 260, wherein the dominant mutation is a dominant neomorphic mutation.

264. The method according to any of claims 254-257, wherein the modulation is mediated by a transcription factor.

265. The method according to any of claims 254-257, wherein the modulation is mediated by a peptide modulator of gene expression.

266. The method according to claim 265, wherein the peptide modulator is an activator of gene expression.

267. The method according to claim 265, wherein the peptide modulator is an inhibitor of gene expression.

268. The method according to any of claims 254-257, wherein the modulation is mediated by a small molecule modulator of gene expression.

269. The method according to claim 268, wherein the small molecule modulator is an activator of gene expression.

270. The method according to claim 268, wherein the small molecule modulator is an inhibitor of gene expression.

271. The method according to any of claims 254-270, wherein the gene acts on a transcription factor.

272. The method according to any of claims 254-270, wherein the gene acts on a transmembrane transporter.

273. The method according to any of claims 254-270, wherein the gene acts on a kinase.

274. The method according to any of claims 254-270, wherein the gene acts on a G-protein.

275. The method according to any of claims 254-270, wherein the gene acts on a phosphatase.

276. The method according to any of claims 254-270, wherein the gene acts on a protease.

277. The method according to any of claims 254-270, wherein the gene acts on a biosynthetic enzyme.

278. The method according to any of claims 254-270, wherein the gene acts on a cell surface receptor.

279. The method according to any of claims 254-270, wherein the gene acts on a GTPase activating protein.

280. The method according to any of claims 254-270, wherein the gene acts on a guanine nucleotide exchange factor.

281. The method according to any of claims 254-270, wherein the gene acts on a cyclic nucleotide phosphodiesterase.

282. The method according to any of claims 254-270, wherein the gene acts on a bacterial protein toxin.

283. The method according to any of claims 254-270, wherein the gene acts on an importin protein.

284. The method according to any of claims 254-270, wherein the gene acts on an RNA binding protein.

285. The method according to any of claims 254-270, wherein the gene acts on a component of a SCF complex.

286. The method according to any of claims 254-270, wherein the gene encodes a transcription factor.

287. The method according to any of claims 254-270, wherein the gene encodes a transmembrane transporter.

288. The method according to claim 287, wherein the transmembrane transporter is a pump.

289. The method according to any of claims 254-270, wherein the gene encodes a kinase.

290. The method according to any of claims 254-270, wherein the gene encodes a G-protein.

291. The method according to any of claims 254-270, wherein the gene encodes a phosphatase.

292. The method according to any of claims 254-270, wherein the gene encodes a protease.

293. The method according to any of claims 254-270, wherein the gene encodes a biosynthetic enzyme.

294. The method according to any of claims 254-270, wherein the gene encodes a cell surface receptor.

295. The method according to any of claims 254-270, wherein the gene encodes a GTPase activating protein.

296. The method according to any of claims 254-270, wherein the gene encodes a guanine nucleotide exchange factor.

297. The method according to any of claims 254-270, wherein the gene encodes a cyclic nucleotide phosphodiesterase.

298. The method according to any of claims 254-270, wherein the gene encodes a bacterial protein toxin.

299. The method according to any of claims 254-270, wherein the gene encodes an importin protein.

300. The method according to any of claims 254-270, wherein the gene encodes an RNA binding protein.

301. The method according to any of claims 254-270, wherein the gene encodes a component of a SCF complex.

302. The method according to any of claims 254-301, wherein the secondary metabolite is an antibacterial.

303. The method according to claim 302, wherein the antibacterial is a ß-lactam.

304. The method according to claim 302, wherein the antibacterial is a cephalosporin.

305. The method according to any of claims 254-301, wherein the secondary metabolite is an anti-hypercholesterolemic.

306. The method according to claim 305, wherein the anti-hypercholesterolemic is selected from lovastatin, mevastatin, simvastatin and pravastatin.

307. The method according to any of claims 254-301, wherein the secondary metabolite is an immunosuppressant.

308. The method according to claim 307, wherein the immunosuppressant is a cyclosporin.

309. The method according to any of claims 254-301, wherein the secondary metabolite is an ergot alkaloid.

310. The method according to any of claims 254-301, wherein the secondary metabolite is an angiogenesis inhibitor.

311. The method according to any of claims 254-301, wherein the secondary metabolite is a glucan synthesis inhibitor.

312. The method according to any of claims 254-301, wherein the secondary metabolite is a fungal toxin.

313. The method according to any of claims 254-301, wherein the secondary metabolite is a gliotoxin.

314. The method according to any of claims 254-301, wherein the secondary metabolite is a modulator of cell surface receptor signaling.

315. The method according to any of claims 254-301, wherein the secondary metabolite is a plant growth regulator.

316. The method according to any of claims 254-301, wherein the secondary metabolite is a pigment.

317. The method according to any of claims 254-301, wherein the secondary metabolite is an insecticide.

318. The method according to any of claims 254-301, wherein the secondary metabolite is an anti-neoplastic compound.

319. The method according to any of claims 254-318, further comprising the step of purifying the secondary metabolite from a culture of the fungus.

320. A method for improving production of a secondary metabolite in a fungus by causing conditional lysis of the fungus, the method comprising modulating the expression of a gene involved in regulation of secondary metabolite production in a manner that causes conditional lysis.

321. The method according to claim 320, wherein the modulation is overexpression of the gene.

322. The method according to claim 320, wherein the modulation is conditional expression of the gene.

323. The method according to claim 320, wherein the modulation is expression of a dominant mutation of the gene.

324. The method according to claim 323, wherein the dominant mutation is a dominant negative mutation.

325. The method according to claim 323, wherein the dominant mutation is a dominant positive mutation.

326. The method according to claim 323, wherein the dominant mutation is a dominant neomorphic mutation.

327. The method according to claim 320, wherein the modulation is mediated by a transcription factor.

328. The method according to claim 320, wherein the modulation is mediated by a peptide modulator of gene expression.

329. The method according to claim 328, wherein the peptide modulator is an activator of gene expression.

330. The method according to claim 328, wherein the peptide modulator is an inhibitor of gene expression.

331. The method according to claim 320, wherein the modulation is mediated by a small molecule modulator of gene expression.

332. The method according to claim 331, wherein the small molecule modulator is an activator of gene expression.

333. The method according to claim 331, wherein the small molecule modulator is an inhibitor of gene expression.

334. The method according to any of claims 320-333, wherein the gene acts on a transcription factor.

335. The method according to any of claims 320-333, wherein the gene acts on a transmembrane transporter.

336. The method according to any of claims 320-333, wherein the gene acts on a kinase.

337. The method according to any of claims 320-333, wherein the gene acts on a G-protein.

338. The method according to any of claims 320-333, wherein the gene acts on a phosphatase.

339. The method according to any of claims 320-333, wherein the gene acts on a protease.

340. The method according to any of claims 320-333, wherein the gene acts on a biosynthetic enzyme.

341. The method according to any of claims 320-333, wherein the gene acts on a cell surface receptor.

342. The method according to any of claims 320-333, wherein the gene acts on a GTPase activating protein.

343. The method according to any of claims 320-333, wherein the gene acts on a guanine nucleotide exchange factor.

344. The method according to any of claims 320-333, wherein the gene acts on a cyclic nucleotide phosphodiesterase.

345. The method according to any of claims 320-333, wherein the gene acts on a bacterial protein toxin.

346. The method according to any of claims 320-333, wherein the gene acts on an importin protein.

347. The method according to any of claims 320-333, wherein the gene acts on an RNA binding protein.

348. The method according to any of claims 320-333, wherein the gene acts on a component of a SCF complex.

349. The method according to any of claims 320-333, wherein the gene encodes a transcription factor.

350. The method according to any of claims 320-333, wherein the gene encodes a transmembrane transporter.

351. The method according to claim 350, wherein the transmembrane transporter is a pump.

352. The method according to any of claims 320-333, wherein the gene encodes a kinase.

353. The method according to any of claims 320-333, wherein the gene encodes a G-protein.

354. The method according to any of claims 320-333, wherein the gene encodes a phosphatase.

355. The method according to any of claims 320-333, wherein the gene encodes a protease.

356. The method according to any of claims 320-333, wherein the gene encodes a biosynthetic enzyme.

357. The method according to any of claims 320-333, wherein the gene encodes a cell surface receptor.

358. The method according to any of claims 320-333, wherein the gene encodes a GTPase activating protein.

359. The method according to any of claims 320-333, wherein the gene encodes a guanine nucleotide exchange factor.

360. The method according to any of claims 320-333, wherein the gene encodes a cyclic nucleotide phosphodiesterase.

361. The method according to any of claims 320-333, wherein the gene encodes a bacterial protein toxin.

362. The method according to any of claims 320-333, wherein the gene encodes an importin protein.

363. The method according to any of claims 320-333, wherein the gene encodes an RNA binding protein.

364. The method according to any of claims 320-333, wherein the gene encodes a component of a SCF complex.

365. The method according to any of claims 320-364, wherein the secondary metabolite is an antibacterial.

366. The method according to claim 365, wherein the antibacterial is a ß-lactam.

367. The method according to claim 365, wherein the antibacterial is a cephalosporin.

368. The method according to any of claims 320-364, wherein the secondary metabolite is an anti-hypercholesterolemic.

369. The method according to claim 368, wherein the anti-hypercholesterolemic is selected from lovastatin, mevastatin, simvastatin and pravastatin.

370. The method according to any of claims 320-364, wherein the secondary metabolite is an immunosuppressant.

371. The method according to claim 370, wherein the immunosuppressant is a cyclosporin.

372. The method according to any of claims 320-364, wherein the secondary metabolite is an ergot alkaloid.

373. The method according to any of claims 320-364, wherein the secondary metabolite is an angiogenesis inhibitor.

374. The method according to any of claims 320-364, wherein the secondary metabolite is a glucan synthesis inhibitor.

375. The method according to any of claims 320-364, wherein the secondary metabolite is a fungal toxin.

376. The method according to any of claims 320-364, wherein the secondary metabolite is a gliotoxin.

377. The method according to any of claims 320-364, wherein the secondary metabolite is a modulator of cell surface receptor signaling.

378. The method according to any of claims 320-364, wherein the secondary metabolite is a plant growth regulator.

379. The method according to any of claims 320-364, wherein the secondary metabolite is a pigment.

380. The method according to any of claims 320-364, wherein the secondary metabolite is an insecticide.

381. The method according to any of claims 320-364, wherein the secondary metabolite is an anti-neoplastic compound.

382. The method according to any of claims 320-381, further comprising the step of purifying the secondary metabolite from a culture of the fungus.

383. A method for improving production of a secondary metabolite in a fungus by increasing the resistance of the fungus to the deleterious effects of exposure to a secondary metabolite, the method comprising modulating the expression of a gene involved in regulation of secondary metabolite production in a manner that increases resistance to the deleterious effects of exposure to a secondary metabolite.

384. The method according to claim 383, wherein the modulation is overexpression of the gene.

385. The method according to claim 383, wherein the modulation is conditional expression of the gene.

386. The method according to claim 383, wherein the modulation is expression of a dominant mutation of the gene.

387. The method according to claim 386, wherein the dominant mutation is a dominant negative mutation.

388. The method according to claim 386, wherein the dominant mutation is a dominant positive mutation.

389. The method according to claim 386, wherein the dominant mutation is a dominant neomorphic mutation.

390. The method according to claim 383, wherein the modulation is mediated by a transcription factor.

391. The method according to claim 383, wherein the modulation is mediated by a peptide modulator of gene expression.

392. The method according to claim 391, wherein the peptide modulator is an activator of gene expression.

393. The method according to claim 391, wherein the peptide modulator is an inhibitor of gene expression.

394. The method according to claim 383, wherein the modulation is mediated by a small molecule modulator of gene expression.

395. The method according to claim 395, wherein the small molecule modulator is an activator of gene expression.

396. The method according to claim 395, wherein the small molecule modulator is an inhibitor of gene expression.

397. The method according to any of claims 383-396, wherein the gene acts on a transcription factor.

398. The method according to any of claims 383-396, wherein the gene acts on a transmembrane transporter.

399. The method according to any of claims 383-396, wherein the gene acts on a kinase.

400. The method according to any of claims 383-396, wherein the gene acts on a G-protein.

401. The method according to any of claims 383-396, wherein the gene acts on a phosphatase.

402. The method according to any of claims 383-396, wherein the gene acts on a protease.

403. The method according to any of claims 383-396, wherein the gene acts on a biosynthetic enzyme.

404. The method according to any of claims 383-396, wherein the gene acts on a cell surface receptor.

405. The method according to any of claims 383-396, wherein the gene acts on a GTPase activating protein.

406. The method according to any of claims 383-396, wherein the gene acts on a guanine nucleotide exchange factor.

407. The method according to any of claims 383-396, wherein the gene acts on a cyclic nucleotide phosphodiesterase.

408. The method according to any of claims 383-396, wherein the gene acts on a bacterial protein toxin.

409. The method according to any of claims 383-396, wherein the gene acts on an importin protein.

410. The method according to any of claims 383-396, wherein the gene acts on an RNA binding protein.

411. The method according to any of claims 383-396, wherein the gene acts on a component of a SCF complex.

412. The method according to any of claims 383-396, wherein the gene encodes a transcription factor.

413. The method according to any of claims 383-396, wherein the gene encodes a transmembrane transporter.

414. The method according to claim 413 wherein the transmembrane transporter is a pump.

415. The method according to any of claims 383-396, wherein the gene encodes a kinase.

416. The method according to any of claims 383-396, wherein the gene encodes a G-protein.

417. The method according to any of claims 383-396, wherein the gene encodes a phosphatase.

418. The method according to any of claims 383-396, wherein the gene encodes a protease.

419. The method according to any of claims 383-396, wherein the gene encodes a biosynthetic enzyme.

420. The method according to any of claims 383-396, wherein the gene encodes a cell surface receptor.

421. The method according to any of claims 383-396, wherein the gene encodes a GTPase activating protein.

422. The method according to any of claims 383-396, wherein the gene encodes a guanine nucleotide exchange factor.

423. The method according to any of claims 383-396, wherein the gene encodes a cyclic nucleotide phosphodiesterase.

424. The method according to any of claims 383-396, wherein the gene encodes a bacterial protein toxin.

425. The method according to any of claims 383-396, wherein the gene encodes an importin protein.

426. The method according to any of claims 383-396, wherein the gene encodes an RNA binding protein.

427. The method according to any of claims 383-396, wherein the gene encodes a component of a SCF complex.

428. The method according to any of claims 383-427, wherein the secondary metabolite is an antibacterial.

429. The method according to claim 428, wherein the antibacterial is a ß-lactam.

430. The method according to claim 428, wherein the antibacterial is a cephalosporin.

431. The method according to any of claims 383-427, wherein the secondary metabolite is an anti-hypercholesterolemic.

432. The method according to claim 431, wherein the anti-hypercholesterolemic is selected from lovastatin, mevastatin, simvastatin and pravastatin.

433. The method according to any of claims 383-427, wherein the secondary metabolite is an immunosuppressant.

434. The method according to claim 433, wherein the immunosuppressant is a cyclosporin.

435. The method according to any of claims 383-427, wherein the secondary metabolite is an ergot alkaloid.

436. The method according to any of claims 383-427, wherein the secondary metabolite is an angiogenesis inhibitor.

437. The method according to any of claims 383-427, wherein the secondary metabolite is a glucan synthesis inhibitor.

438. The method according to any of claims 383-427, wherein the secondary metabolite is a fungal toxin.

439. The method according to any of claims 383-427, wherein the secondary metabolite is a gliotoxin.

440. The method according to any of claims 383-427, wherein the secondary metabolite is a modulator of cell surface receptor signaling.

441. The method according to any of claims 383-427, wherein the secondary metabolite is a plant growth regulator.

442. The method according to any of claims 383-427, wherein the secondary metabolite is a pigment.

443. The method according to any of claims 383-427, wherein the secondary metabolite is an insecticide.

444. The method according to any of claims 383-427, wherein the secondary metabolite is an anti-neoplastic compound.

445. The method according to any of claims 383-444, further comprising the step of purifying the secondary metabolite from a culture of the fungus.

446. A genetically modified fungus, wherein the genetically modified fungus has an ability to produce secondary metabolites and the ability of the genetically modified fungus to produce secondary metabolites has been improved by any of the methods of claims 1-445.

447. A method for making a secondary metabolite, the method comprising culturing a genetically modified fungus according to claim 446 under conditions suitable for the production of secondary metabolites.

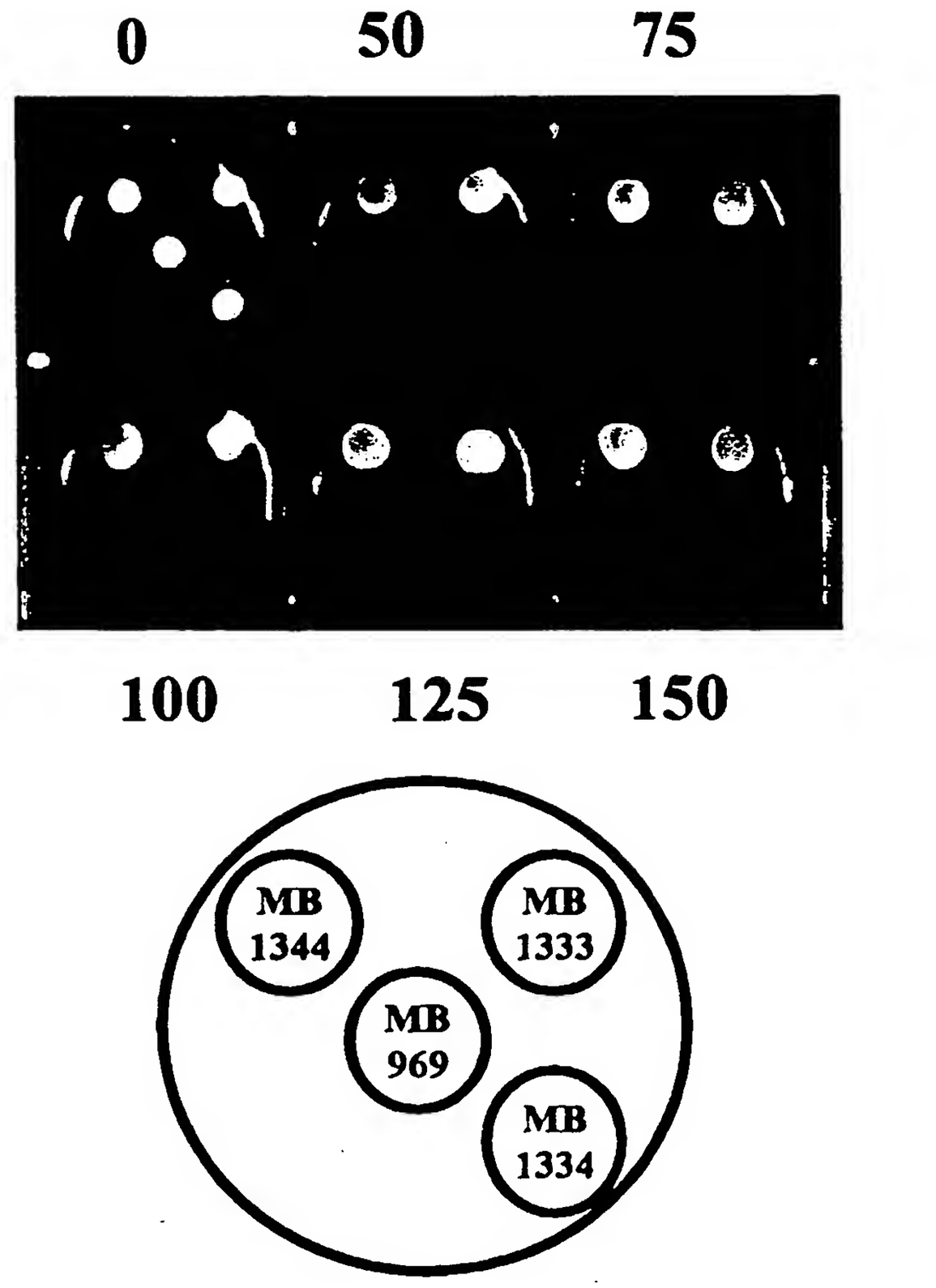

FIG. 1

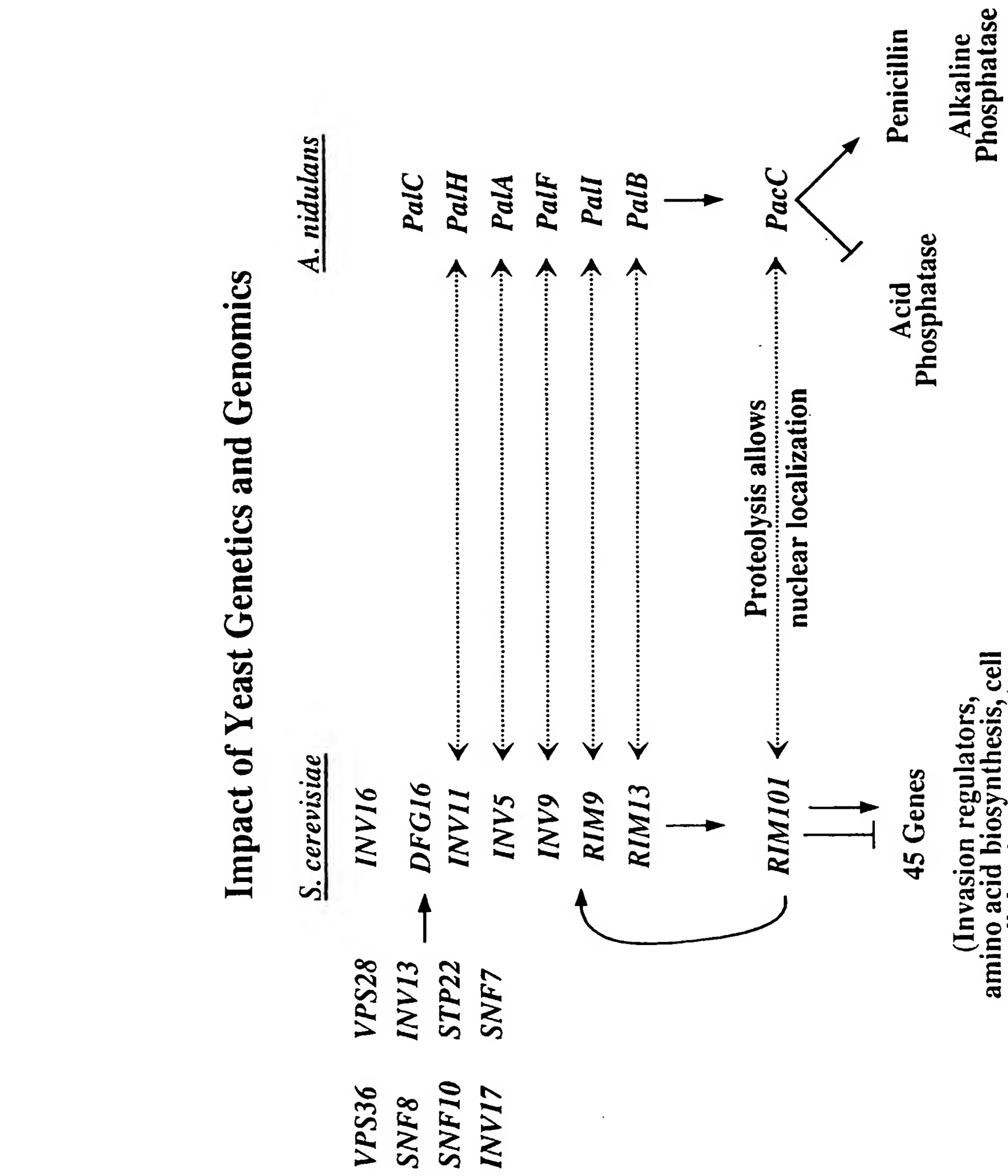

FIG. 2

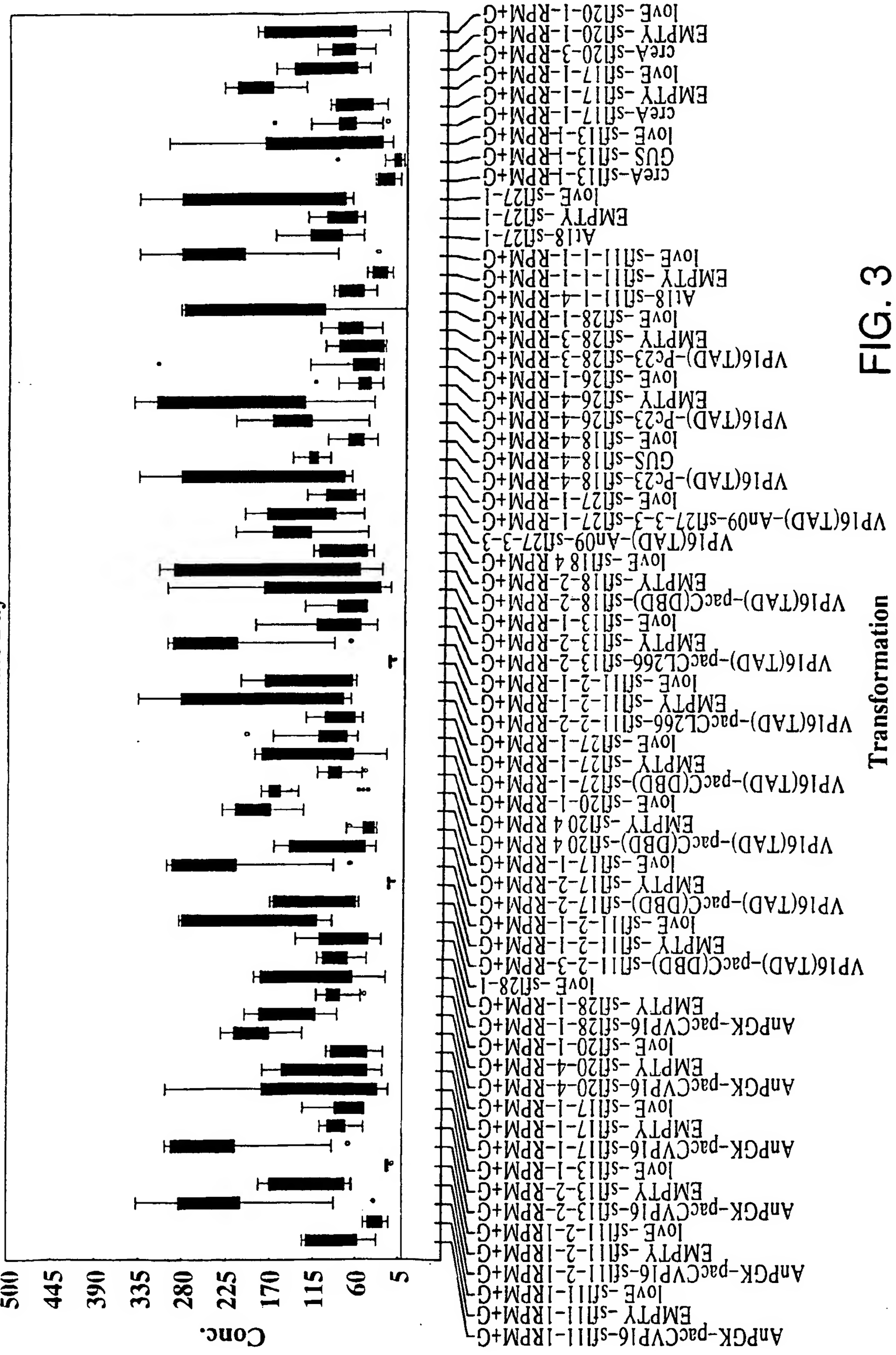

FIG. 3

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/28903

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/37 C12N15/80 C12P21/00 C12R1/645

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C12N C12P C12R

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 25735 A (MICROBIA INC) 27 May 1999 (1999-05-27) the whole document ---	1,446, 447
X	WO 99 25865 A (MICROBIA INC) 27 May 1999 (1999-05-27) the whole document ---	1,446, 447
X	EP 0 357 119 A (GIST BROCADES NV) 7 March 1990 (1990-03-07) the whole document, in particular page 8 line 52 to page 9 lines 3 ---	1,446, 447
X	US 5 665 543 A (LEICHTFRIED FRANZ ET AL) 9 September 1997 (1997-09-09) the whole document, in particular columns 31-34 --- -/--	1,446, 447

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

'A' document defining the general state of the art which is not considered to be of particular relevance

'E' earlier document but published on or after the international filing date

'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

'O' document referring to an oral disclosure, use, exhibition or other means

'P' document published prior to the international filing date but later than the priority date claimed

'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

'&' document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 March 2001	21.03.01

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Julia, P

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/28903

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 00 37629 A (WISCONSIN ALUMNI RES FOUND) 29 June 2000 (2000-06-29) cited in the application the whole document -----	1,446, 447

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 00/28903

Box I Observations where certain claims were found unsearchable (Continuati n of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 2-445 (complete); 1, 446-447 (partially)
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 2-445 (complete); 1, 446-447 (partially)

In view of the large number of claims present on file as well as their wording, the International Search Agency considers that it is difficult, if not impossible, to determine the matter for which protection is actually sought and that the present application fails to comply with the clarity and conciseness requirements of Article 6 PCT (see also Rule 6.1(a) PCT) to such an extent that a meaningful search is impossible.

Moreover, the claims relate to methods defined only by reference to a desirable characteristic, namely "... modulating the expression of a gene involved in regulation of secondary metabolite production in a manner..." that (i) improves the yield of the secondary metabolite (claims 1-64), (ii) improves the productivity of the secondary metabolite (claims 65-127), (iii) increases the flux or excretion of the secondary metabolite, (claims 128-190), (iv) decreases the production of side products or competing secondary metabolites (claims 191-253), (v) alters the characteristics of the fungus (claims 254-319), (vi) causes conditional lysis (claims 320-382) and (vii) increases resistance to the deleterious effects of exposure to a secondary metabolite (claims 383-445) as well as to related products (genetically modified fungi, claim 446) and uses thereof (method of making a secondary metabolite, claim 447). The claims cover all methods having this characteristic, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such methods. In the present case, the claims so lack support and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the methods by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible.

Furthermore, according to Rule 13 PCT an application must relate to one invention only or to a group of inventions so linked as to form a single general inventive concept, i.e. having at least one common technical feature defining a contribution over the known prior art. In the present case, the common technical feature among the different groups of inventions seems to be "...the modulation of the expression of a gene involved in the regulation of secondary metabolite production ... for improving the production of a secondary metabolite by a fungus ...". However, this concept was already known in the prior art (see disclaimers in claim 1, bibliographic references in the description of the application as well as the partial ISR). Therefore, in principle seven different groups of inventions could be identified corresponding to the seven independent claims of the present application, namely methods for improving the production of a secondary metabolite modulating the expression of a gene involved in the regulation of secondary metabolite production in a manner that : (i) improves the yield of the secondary metabolite (claims 1-64), (ii) improves the productivity of the secondary metabolite (claims 65-127), (iii) increases efflux or excretion of the

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secondary metabolite (claims 128-190), (iv) decreases the production of side products or competing secondary metabolites (claims 191-253), (v) alters the characteristics of the fungus (claims 254-319), (vi) causes conditional lysis (claims 320-382), (vii) increases resistance to the deleterious effects of exposure to a secondary metabolite (claims 383-445) as well as related genetically modified fungi and uses thereof for each group (claims 446-447, partially). However, in view of the references cited in the application and in the partial ISR, the ISA fails to see which is the single inventive concept among the different embodimens corresponding to the depent claims of each group of inventions. Thus, each and every group identified above comprises several subgroups of inventions, such as : (i) type of modulation and/or modulator, (ii) type and effects of the genes involved in the regulation of the secondary metabolite production, (iii) type of secondary metabolite, etc...

In conclusion, the claims do not fulfil the requirements of Articles 5 and 6 PCT and Rule 13 PCT. A meaningful search over the whole of the claimed scope is impossible and consequently, the search has been carried out only for those parts of the application which do appear to be (i) clear, concise, technically supported and (ii) which correspond to the first group of invention (claims 1 and 446-447, partially), namely those parts relating to methods for increasing the yield of a secondary metabolite using the "fungal regulators" of Table 1 (examples 3-5). However, in view of the document WO99/25735 no common inventive concept seems to be present among these several fungal regulators.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

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